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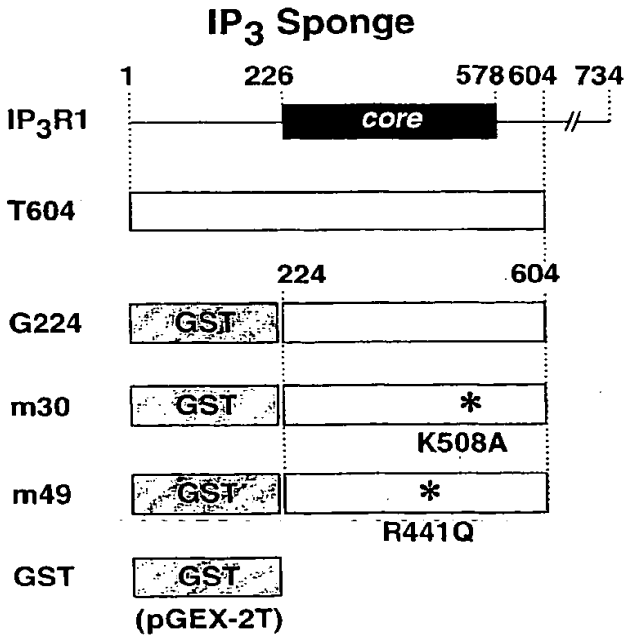
(54) High affinity IP3-binding polypeptide

(57) The present invention provides a high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate, to a gene encoding the polypeptide, to a recombinant vector including the gene, to a transformant including the vector and to a method for producing the high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate.

FIG. 1



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Description

FIELD OF THE INVENTION

[0001] The present invention relates to a high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate, to a gene encoding the polypeptide, to a recombinant vector including the gene, to a transformant including the vector and to a method for producing the high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate.

BACKGROUND OF THE INVENTION

[0002] Inositol 1,4,5-trisphosphate (hereinafter, also referred to as "IP_3") is one of second messengers which are produced by inositol phospholipid metabolism activated in response to an extracellular stimului such as hormones, growth factors, neurotransmitters or the like. IP_3 is a substance that induces the increase of intracellular calcium concentration. The IP_3-induced calcium increase is a crucial and highly universal signal transmission mechanism that is involved in many cell functions in a wide variety of animals. For example, IP_3 controls many physiological functions such as fertilization, blastogenesis, development and differentiation, cell growth, secretion, immune system, muscle contraction, and cranial nerve functions (gustation, vision, memory, learning, etc.) in diverse organisms, for example, invertebrata such as nematoda (nemathelminthes), Drosophila (arthropoda) and cuttlefish (mollusca), and vertebrata such as mouse and human.

[0003] On the molecular level, this mechanism is initiated by the binding between an IP_3 and its target, an IP_3 receptor. Specifically, when the IP_3 binds to the IP_3 receptor (a calcium channel susceptible to IP_3) present in an intracellular calcium-storing site (endoplasmic reticulum, etc.), the channel opens and releases calcium from the calcium-storing site into the cytoplasm, thereby controlling the activities of calcium-dependent proteins and enzymes.

[0004] Heparin, adenophostin (a kind of fungal metabolite) and Xestospongin (a kind of sponge metablite) are examples of substances that might affect the signal transmission by the IP_3-induced calcium. However, although heparin inhibits the binding between the IP_3 and the IP_3 receptor, its specificity is low since there are various targets in the cell. Adenophostin is an antagonistic agonist of the binding between the IP_3 and the IP_3 receptor, and is a powerful activator of the IP_3 receptor channel. However, its use is limited since its yield from fungus is low and it cannot transport across the membrane. Xestospongin has recently been reported as an inhibitor of the IP_3 receptor channel that does not influence the binding of IP_3. Again, its yield is low and there are still questions remaining as to its specificity. Thus, currently, there is almost no substance that is considered to effectively act on IP_3-induced calcium signal transmission. In particular, there has been no substance or system that inhibits IP_3-induced calcium signal transmission by specifically trapping IP_3 that has increased on the cell level.

SUMMARY OF THE INVENTION

[0005] The present invention provides a high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate, a gene encoding the polypeptide, a recombinant vector containing the gene, a transformant containing the vector and a method for producing the high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate.

[0006] In order to solve the above-described problem, the present inventors have gone through intensive studies and have succeeded in isolating a high affinity polypeptide having an extremely high binding activity to IP_3 from a protein including a part of the N-terminal amino acid region of an IP_3 receptor.

[0007] The present invention provides a recombinant polypeptide of the following (a), (b) or (c):

(a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO: 2;
(b) a polypeptide comprising an amino acid sequence having deletion, substitution or addition of at least one amino acid in the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity to inositol 1,4,5-trisphosphate; or
(c) a polypeptide having at least 70% homology with the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity with inositol 1,4,5-trisphosphate.

[0008] The present invention also provides a gene coding for a polypeptide of the above (a), (b) or (c); or a gene coding for a polypeptide having at least 70% homology with the gene and having a high affinity binding activity with inositol 1,4,5-trisphosphate.

[0009] The present invention further provides a gene comprising DNA of the following (d) or (e):

(d) DNA of a nucleotide sequence shown in SEQ ID NO: 1; or
(e) DNA of a nucleotide sequence having at least 70% homology with the DNA of the nucleotide sequence shown

in SEQ ID NO: 1, and coding for a polypeptide having a high affinity binding activity with inositol 1,4,5-trisphosphate.

[0010] The present invention provides a recombinant vector comprising any one of the above-described genes.

[0011] The present invention also provides a transformant comprising the above recombinant vector.

[0012] The present invention further provides a method for producing any one of the above-mentioned polypeptides, the method comprising: culturing the above-mentioned transformant; and collecting, from the obtained culture, a polypeptide having a high affinity binding activity to inositol 1,4,5-trisphosphate.

[0013] This and other advantages of the present invention will become apparent to those skilled in the art upon reading and understanding the following detailed description with reference to the accompanying figures.

[0014] This specification includes part or all of the contents as disclosed in the specification and/or drawings of Japanese Patent Application No. 10-242207 which is a priority document of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

Fig. 1 shows the structures of IP_3 sponges;

Figs. 2A-2C show high expression and IP_3-binding activity of T604;

Figs. 3A-3C are graphs showing the IP_3-binding activities of the IP_3 sponges;

Fig. 4 is a graph showing a curve of IP_3-binding inhibition depending on the IP_3 sponge concentration;

Figs. 5A-5F are graphs showing the effects of low-affinity G224-m30 and GST on IP_3-induced Ca^{2+} release;

Figs. 6A-6G are graphs showing the effect of high affinity IP_3 sponge G224 on inhibition of IP_3-induced Ca^{2+} release; and

Fig. 7 is a plot diagram showing an IP_3-induced Ca^{2+} release depending on the concentration of the high affinity IP_3 sponge G224.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0016] Hereinafter, the present invention will be described in more detail.

[0017] A polypeptide of the present invention specifically binds to IP_3 with a very high affinity, and includes a part (a cut) of N-terminal amino acid region of the natural IP_3 receptor (thus also referred to as a cut-type polypeptide). The polypeptide of the invention is often referred to as a high affinity IP_3-binding polypeptide.

1. Cloning a gene coding for the IP_3 receptor

[0018] In order to obtain a high affinity IP_3-binding polypeptide of the invention, a gene encoding the natural IP_3 receptor protein is cloned. The nucleotide sequence of the IP_3 receptor gene is already known (*Nucleic Acid Res,* 17: 5385-5386, 1989; *Nature* 342:32-38, 1989). The gene may, for example, be prepared according to the following gene engineering procedure.

(i) Preparation and screening of cDNA library encoding the IP_3 receptor

[0019] A known procedure may be employed to prepare mRNA of the IP_3 receptor. For example, total RNA is obtained by treating a tissue or a cell from a mouse brain with a guanidine reagent, a phenol reagent or the like. Then, poly(A) +RNA(mRNA) is obtained according to an affinity column method or a batch method using poly (U) sepharose, etc. By using the obtained mRNA as a template as well as oligo dT primer and reverse transcriptase, a single-stranded cDNA is synthesized. Based on the single-stranded cDNA, a doublestranded cDNA is synthesized and introduced into a suitable cloning vector to prepare a recombinant vector to transform *E.coli* or the like. The transformant is selected based on indices such as tetracycline and ampicillin resistance, thereby obtaining a cDNA library.

[0020] The transformation of *E.coli* may be conducted according to the method of Hanahan [Hanahan, D., *J. Mol. Biol.* 166:557-580 (1983)]. Specifically, the recombinant vector is added to a prepared competent cell under the presence of calcium chloride, magnesium chloride or rubidium chloride. When a plasmid is used as the vector, it should

contain a gene resistant to drugs such as tetracycline and ampicillin. Besides plasmids, a cloning vector such as λ phage may also be used.

[0021] The thus-obtained transformant is screened for strains with the DNA of interest by, for example, "expression cloning" through immunoscreening using an antibody, or by polymerase chain reaction (PCR) using a primer synthesized from a known sequence.

[0022] The thus-obtained DNA fragment or DNA amplified fragment coding for the antibody epitope is labeled with ^{32}P, ^{35}S, biotin or the like to be used as a probe for hybridizing with the transformant DNA denatured and bound on a nitrocellrose filter. Then, the obtained positive strains may be screened for the target DNA fragment.

(ii) Determination of the nucleotide sequence

[0023] The obtained clone is determined for its nucleotide sequence. The nucleotide sequence may be determined according to a known method such as Maxam-Gilbert chemical modification method, dideoxynucleotide chain termination method using M13 phage. Generally, the sequence is determined using an automatic DNA sequencer (e.g., Perkin-Elmer 373A DNA sequencer).

[0024] The nucleotide sequence of the natural (full-length) gene coding for the IP_3 receptor and the full-length amino acid sequence of the IP_3 receptor are shown in SEQ ID NOS. 3 and 4, respectively.

2. Design and synthesis of a gene coding for a high affinity IP_3-binding polypeptide of the invention

(i) Design and synthesis of a gene coding for a high affinity IP_3-binding polypeptide

[0025] A high affinity IP_3-binding polypeptide of the invention includes a cut of N-terminal amino acid region, that is, Amino acids 579 to at least 800, preferably Amino acids 579 to at least 734, of the amino acid sequence of the full-length IP_3 receptor protein (SEQ ID NO:4). According to the present invention, this cut-type polypeptide is also referred to as an IP_3 sponge (Fig. 1). Due to this cut, the polypeptide (IP_3 sponge) of the invention gains a very strong specific binding ability to IP_3 (high affinity IP_3-binding activity).

[0026] Herein, the phrase "high affinity" is used in the situation where the IP_3 sponge has an IP_3 affinity that is about 100 to 1,000 times (preferably 500 to 1,000 times) higher than that of the natural IP_3 receptor.

[0027] According to the present invention, the IP_3 sponge also includes at least the amino acid sequence shown in SEQ ID NO: 2, which corresponds to Amino acids 226-578 of the amino acid sequence of SEQ ID NO: 4. Herein, this region is referred to as a "core" region.

[0028] Based on the above-described facts, the length of the fragment of the invention and the length of the DNA coding for the fragment can be determined at ones discretion providing that the high affinity IP_3-binding activity is maintained. The fragment may include, for example, Amino acids 224-604 of the amino acid sequence of SEQ ID NO: 4 (encoded by Nucleotides 670-1812 of the nucleotide sequence of SEQ ID NO: 3); Amino acids 1-604 of the amino acid sequence of SEQ ID NO: 4 (encoded by Nucleotides 1-1812 of the nucleotide sequence of SEQ ID NO: 3); or Amino acids 1-734 of the amino acid sequence of SEQ ID NO: 4 (encoded by Nucleotides 1-2200 of the nucleotide sequence of SEQ ID NO: 3).

[0029] These fragments are obtained through PCR using primers that are designed based on nucleotide regions of the nucleotides shown in SEQ ID NO. 3 outside the regions of the respective fragments, as well as the DNA coding for the natural IP_3 receptor (SEQ ID NO: 3, *Nucleic Acid Res.* 17: 5385-5386, 1989; *Nature* 342: 32-38, 1989) as a template.

(ii) Preparation of a gene encoding a mutant-type IP_3 sponge of the invention (mutant-type IP_3 gene)

[0030] According to the present invention, the amino acid sequence of the IP_3 sponge may, at least partially, be introduced with a mutation. Such a mutant-type IP_3 sponge is also contemplated as the IP_3 sponge of the present invention. A mutation is introduced into the amino acid sequence, by mutating the nucleotide sequence of the gene coding for the amino acid sequence of the IP_3 sponge.

[0031] The mutation is introduced into the gene according to a known method such as Kunkel method, Gapped duplex method or any method equivalent thereof. For example, site-directed mutatagenesis may be employed in which a mutant oligonucleotide is used as a primer (Yoshikawa, F. et al., *J. Biol. Chem.* 271: 18277-18284, 1996). Alternatively, a mutation may be introduced by using a mutagenesis kit such as Mutant-K (Takara), Mutant-G (Takara) and a series of LA PCR *in vitro* Mutagenesis kits (Takara).

[0032] First, based on the nucleotides of the gene coding for the IP_3 sponge of the invention (also referred to as an "IP_3 sponge gene"), a primer is synthesized such that the primer includes a mutated nucleotide or site and about 10 nucleotides flanking the mutated nucleotide or site. Using this primer as well as the IP_3 sponge gene as a template,

PCR reaction is conducted. The resultant is purified and then treated with a suitable restriction enzyme, thereby obtaining the mutant-type IP_3 sponge gene of interest.

(iii) Determination of the nucleotide sequences

[0033] The nucleotide sequence of the genes obtained through (i) and (ii) is determined. The determination is conducted by a known method such as Maxam-Gilbert chemical modification method, dideoxynucleotide chain termination method using M13 phage, or any other method. Generally, an automatic sequencer (e.g., 373A DNA sequencer produced by Perkin-Elmer) is used.

[0034] A nucleotide sequence of an IP_3 sponge gene of the invention and an amino acid sequence of the IP_3 sponge of the invention are shown in SEQ ID NOS: 1 and 2, respectively. The polypeptide of this amino acid sequence may include at least one deletion, substitution, addition or the like as long as it has a high affinity with IP_3 and has an activity of specifically binding to IP_3.

[0035] For example, at least one, preferably about 1 to 10, more preferably 1 to 5 of the amino acids in the core region (the amino acid sequence shown in SEQ ID NO: 2) may be deleted; at least one, preferably about 1 to 10, more preferably 1 to 5 amino acids may be added to the amino acid sequence of the core region; or at least one, preferably 1 to 10, more preferably 1 to 5 of the amino acids in the core region may be replaced with other amino acids.

[0036] The polypeptide of the present invention is not limited by the length of the amino acid sequence as long as the amino acid sequence contains the amino acid sequence of the core region, and a cut of N-terminal Amino acids 579 to at lease 800, preferably N-terminal Amino acids 579 to at least 734 of the natural-type IP_3 receptor (SEQ ID NO: 4). For example, Amino acids 224-604 (polypeptide "G224") of the amino acid sequence shown in SEQ ID NO: 4, and the gene encoding G224 are also contemplated as the IP_3 sponge and the IP_3 sponge gene of the invention, respectively.

[0037] The polypeptide G224 may have a mutation of at least one, preferably about 1 to 10, more preferably 1 to 5 amino acids. Thus, the IP_3 sponge of the invention may include an amino acid sequence where Lysine at Position 508 of the amino acid sequence G244 is replaced with alanine (mutation "m30") or where arginine at Position 441 of the amino acid sequence G244 is replaced with glutamin (mutation "m49") (Figure 1). Herein, the numbers indicating the positions of the amino acids are based on the amino acid sequence shown in SEQ ID NO: 4 (e.g., Position 1 is the first amino acid of SEQ ID NO: 4).

[0038] A polypeptide including an amino acid sequence having 70% or more homology with the core region (SEQ ID NO: 2), and having a high affinity binding activity with inositol 1,4,5-trisphosphate is also contemplated as the present invention.

[0039] Also contemplated as the present invention is a gene coding for the polypeptide having the above-described mutation in its amino acid sequence, and having a high affinity binding activity with IP_3 receptor. In addition, a nucleotide sequence coding for the amino acids included in the IP_3 sponge of the present invention, and a degenerate isomer coding for the same polypeptide with different degenerate codons are also contemplated as the genes of the invention. Also contemplated as the present invention is DNA having at least 70% homology with the nucleotide sequence of these genes, for example, DNA of other type belonging to the IP_3 receptor gene family that codes for a region corresponding to the polypeptide of the present invention.

[0040] Once the nucleotide sequence of the gene of the present invention is determined, the gene may be obtained by PCR using a primer that is synthesized chemically or that is synthesized from the determined nucleotide sequence.

3. Preparation of recombinant vector and transformant containing IP_3 sponge gene of the invention

(i) Preparation of recombinant vector

[0041] A recombinant vector of the invention may be obtained by ligating (inserting) the IP_3 sponge gene of the invention to (into) a suitable vector. The vector for inserting the gene of the invention is not limited to a specific one as long as it is replicable in a host cell. Examples of such vector include but not limited to plasmid DNA and phage DNA.

[0042] The plasmid DNA is, for example, plasmid from *E.coli* (e.g., pET-3a, pBR322, pBR325, pUC118, pUC119, etc.), plasmid from bacillus (e.g., pUB110, pTP5, etc.), or plasmid from yeast (e.g., YEpl3, YEp24, YCp50, etc.). The phage DNA is, for example, λ phage. Similarly, an animal virus vector such as retrovirus, adenovirus or vaccinia virus vectors, or an insect virus vector such as a baculovirus vector may also be used. A fusion plasmid in which GST, GFP, His-tag, Myc-tag or the like is linked with each other may also be used (e.g., pGEX-2T, pEGFP-N3).

[0043] To insert the gene of the invention into the vector, first, the purified DNA is cleaved with suitable restriction enzymes. Then, the cleaved fragment is inserted into a restriction site or a multicloning site of the suitable vector DNA.

[0044] The gene of the present invention should be integrated into the vector such that the gene can function. If desired, the vector of the invention may include, other than the gene of the invention and the promoter, for example,

a cis-element (e.g., an enhancer), a splicing signal, a poly(A) tail signal, a selective marker, and a ribosome binding sequence (SD sequence). Examples of the selective marker include a dihydrofolate reductase gene, an ampicillin-resistant gene and a neomycin-resistant gene.

(ii) Preparation of transformant

[0045] A transformant of the invention may be obtained by introducing the recombinant vector of the invention into a host cell in such a manner that the gene of interest is capable to be expressed. The host cell is not limited to a specific one as long as it can express the gene of the present invention. Bacteria such as genus *Escherichia* (e.g., *Escherichia coli*), genus *Bacillus* (e.g., *Bacillus subtilis*), genus *Pseudomonas* (e.g,. *Pseudomonas putida*), yeast such as *Saccharomyces* cerevisiae and *Schizosaccharomyces pombe,* animal cells (e.g., COS, CHO, HEK293, PC12 cells), and insect cells (e.g., Sf9 and Sf21) are exemplified.

[0046] When a bacterium such as *E.coli* is used as the host, it is preferable that the recombinant vector of the present invention is capable of autonomous replication in the host and that it includes a promoter, a ribosome binding sequence, the gene of the invention and a transcription termination sequence. The recombinant vector may also include a gene for controlling the promoter.

[0047] As the *E.coli, E. coli* BL21, JM109 and HB101 are exemplified and as bacillus, *Bacillus subtilis* MI 114 and 207-21 are exemplified.

[0048] Any promoter may be used as long as it can be expressed in a host cell like *E.coli,* For example, a promoter derived from *E.coli* or phage, e.g., trp promoter, lac promoter, p_L promoter or p_R promoter, may be used. Artificially designed and modified promoter like tac promoter may also be used.

[0049] The recombinant vector may be introduced into the host bacterium according to any method for introducing DNA into a bacterium. For example, calcium ion method (Cohen, S.N. et al., *Proc. Natl. Acad. Sci.*, USA, 69: 2110-2114 (1972)) and an electroporation method may be employed.

[0050] An yeast such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe* or *Pichia pastoris* may also be used as the host. In this case, the promoter may be any promoter that can be expressed in the yeast. Examples of such promoter include but not limited to gal1 promoter, gal10 promoter, heat shock protein promoter, MF 1 promoter, PHO5 promoter, PGK promoter, GAP promoter, ADH promoter and AOX1 promoter.

[0051] The recombinant vector may be introduced into the yeast by any method for introducing DNA into an yeast. For example, electroporation method (Becker, D.M. et al., *Methods Enzymol.,* 194, 182-187 (1990)), spheroplast method (Hinnen, A. et al., *Proc. Natl. Acad. Sci.,* USA, 75, 1929-1933 (1978)), or lithium acetate method (Itoh, H., *J. Bacteriol.,* 153, 163-168 (1983)) may be employed.

[0052] An animal cell such as simian cell (e.g., COS-7, Vero), Chinese hamster ovary cell (CHO cell), mouse L cell, rat cell (e.g., GH3, PC12 or NG108-15) or human cell (e.g., FL, HEK293, HeLa or Jurkat) may also be used as the host. As a promoter, for example, SR promoter, SV40 promoter, LTR promoter or β-actin promoter may be used. Other than these promoters, an early gene promoter of human cytomegalovirus may also be used.

[0053] The recombinant vector may be introduced into the animal cell, for example, by an electroporation method, a calcium phosphate method or a lipofection method.

[0054] An insect cell such as Sf9 cell, Sf21 cell or the like may also be used as the host. The recombinant vector may be introduced into the insect cell, for example, by a calcium phosphate method, a lipofection method or an electroporation method.

4. Production of IP_3 sponge

[0055] The IP_3 sponge of the present invention may be obtained by culturing the above-described transformant, and recovering the IP_3 sponge from the culture product. The term "culture" as used herein refers to a culture supernatant, a cultured cell or microbial cell, or a cell or microbial cell debris.

[0056] The transformant of the invention is cultured according to a general method employed for culturing the host.

[0057] A medium for culturing the transformant obtained from a microorganism host such as *E.coli* or yeast may be either a natural or a synthetic medium providing that it contains carbon sources, nitrogen sources, inorganic salts and the like assimilable by the microorganism, and that it can efficiently culture the transformant.

[0058] As carbon sources, carbohydrate such as glucose, fructose, sucrose, starch; organic acids such as acetic acid, propionic acid; and alcohols such as ethanol and propanol may be used.

[0059] As nitrogen sources, ammonia; ammonium salts of inorganic or organic acids such as ammonium chloride, ammonium sulfate, ammonium acetate and ammonium phosphate; other nitrogen-containing compounds; Peptone; meat extract; corn steep liquor and the like may be used.

[0060] As inorganic substances, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, iron(II) sulfate, manganese sulfate, copper sulfate, calcium carbonate

and the like may be used.

[0061] The cultivation is generally performed under aerobic conditions such as shaking or aeration agitating conditions at 37°C for 6 to 24 hours. During the cultivation, pH is maintained at 7.0 to 7.5. pH is regulated with an inorganic or organic acid, an alkali solution or the like. If necessary, an antibiotic such as ampicillin, tetracycline or the like may be added to the medium during the cultivation.

[0062] When culturing a microorganism transformed with an expression vector using an inducible promoter, an inducer may be added to the medium at need. For example, isopropyl 1-thio-β-D-galactoside (IPTG) may be added to the medium when culturing a microorganism transformed with an expression vector pET-3a having T7 promoter (that is inducible with IPTG). When culturing a microorganism transformed with an expression vector using trp promoter (that is inducible with indole acetic acid (IAA)), IAA may be added to the medium.

[0063] A transformant obtained with an animal cell host may be cultured in a generally used medium such as RPMI1640 medium or DMEM medium, or a medium obtained by supplementing the generally used medium with fetal bovine serum and the like.

[0064] The cultivation is generally conducted under 5% CO_2 at 37°C for 1 to 30 days. If necessary, an antibiotic such as kanamycin, penicillin or the like may be added to the medium during the cultivation.

[0065] After the cultivation, in the case where a microbial cell or a cell intracelluraly produced the IP_3 sponge of the invention, the IP_3 sponge is collected by disrupting the microbial cell or the cell by sonication, freezing and thawing method, or homogenizing. In the case where a microbial cell or a cell extracellularly produced the IP_3 sponge of the invention, the microbial cell or the cell is removed from the culture through centrifugation or the like before, or the culture solution is directly subjected to the isolation/purification procedure. The IP_3 sponge of the invention is isolated and purified from the culture through a general biochemical method for isolating and purifying a protein, such as ammonium sulfate precipitation, gel chromatography, ion exchange chromatography, affinity chromatography, or a combination thereof.

5. Therapeutic Agent and Agent for Gene Therapy

[0066] Since the protein and the gene of the invention has IP_3 neutralizing activity, they are useful as an antagonist for IP_3-induced calcium, a therapeutic agent and an agent for gene therapy for diseases associated with calcium production. The therapeutic agent or the agent for gene therapy of the invention can be administered orally or parenterally and systemically or locally.

[0067] When the protein or the gene of the invention is used as a therapeutic agent or an agent for gene therapy for disease associated with calcium production, the disease to be treated is not particularly limited. For example, the protein or the gene may be used for diseases in the nervous system, blood vascular system, respiratory system, digestive system, lymphatic system, urinary system, reproduction system or the like for the specific purpose of treatment or prevention. These diseases may be in the form of a single disease or may be complicated by one of these diseases or by some disease other than those mentioned above; any of such forms may be treated with the protein or the gene of the invention.

[0068] When the therapeutic agent of the invention is administered orally, the agent may be formulated into a tablet, capsule, granule, powder, pill, troche, internal liquid agent, suspension, emulsion, syrup or the like. Alternatively, the therapeutic agent may be prepared into a dry product which is re-dissolved just before use. When the therapeutic agent of the invention is administered parenterally, the agent may be formulated into a intravenous injection (including drops), intramuscular injection, intraperitoneal injection, subcutaneous injection, suppository, or the like. Injections are supplied in the form of unit dosage ampules or multi-dosage containers.

[0069] These formulations may be prepared by conventional methods using appropriate excipients, fillers, binders, wetting agents, disintegrating agents, lubricating agents, surfactants, dispersants, buffers, preservatives, dissolution aids, antiseptics, flavoring/perfuming agents, analgesics, stabilizers, isotonicity inducing agents, etc. conventionally used in pharmaceutical preparations.

[0070] Each of the above-described formulations may contain pharmaceutically acceptable carriers or additives. Specific examples of such carriers or additives include water, pharmaceutically acceptable organic solvents, collagen, polyvinyl alcohol, polyvinylpyrrolidone, carboxyvinyl polymers, sodium alginate, water-soluble dextran, sodium carboxymethyl amylose, pectin, xanthan gum, gum arabic, casein, gelatin, agar, glycerol, propylene glycol, polyethylene glycol, vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin, mannitol, sorbitol and lactose. One or a plurality of these additives are selected or combined appropriately depending of the form of the preparation.

[0071] The dosage levels of the therapeutic agent of the invention will vary depending on the age of the subject, the route of administration and the number of times of administration and may be varied in a wide range. When an effective amount of the protein of the invention is administered in combination with an appropriate diluent and a pharmaceutically acceptable carrier, the effective amount of the protein can be in the range from 0.0001 to 1000 mg/kg per administration. The therapeutic agent is administered once a day or in several dosages per day for at least one day.

[0072] When the gene of the invention is used as an agent for gene therapy for diseases associated with calcium production, the gene of the invention may be directly administered by injection. Alternatively, a vector incorporating the gene of the invention may be administered. Specific examples of a suitable vector for this purpose include an adenovirus vector, adeno-associated virus vector, herpes virus vector, vaccinia virus vector and retrovirus vector. The gene of the invention can be administered efficiently by using such a virus vector. Alternatively, the gene of the invention may be enclosed in phospholipid vesicles such as liposomes, and the resultant liposomes may be administered to the subject. Briefly, since liposomes are biodegradable material-containing closed vesicles, the gene of the invention is retained in the internal aqueous layer and the lipid bilayer of liposomes by mixing the gene with the liposomes (a liposome-gene complex). Subsequently, when this complex is cultured with cells, the gene in the complex is taken into the cells (lipofection). Then, the resultant cells may be administered by the methods described below.

[0073] As a method for administering the agent for gene therapy of the invention, local administration to tissues of the central nervous system (brain, spiral cord), blood vascular system (artery, vein, heart), respiratory system (trachea, lung), digestive system (salivary glands, stomach, intestines, liver, pancreas), lymphatic system (lymph node, spleen, thymus), urinary system (kidney), reproduction system (testis, ovary, uterus) or the like may be performed in addition to conventional systemic administration such as intravenous or intra-arterial administration. Further, an administration method combined with catheter techniques and surgical operations may also be employed.

[0074] The dosage levels of the agent for gene therapy of the invention vary depending on the age, sex and conditions of the subject, the route of administration, the number of times of administration, and the type of the formulation. Usually, it is appropriate to administer the gene of the invention in an amount of 0.01-100 mg/adult body/day.

EXAMPLES

[0075] Hereinafter, the present invention will be described in detail by way of examples which do not limit the technical scope of the present invention.

Example 1: Construction of Expression Plasmid for High Affinity IP_3-Binding Polypeptide (IP_3 Sponge)

[0076] The N-terminal amino acids (734 amino acids) (polypeptide T734) of a mouse Type-1 IP_3 receptor (mIP_3R1) has a specific IP_3-binding activity. The cDNA portion coding for polypeptide T734 was cloned into *E.coli* expression vector pET-3a (whose expression is controlled by T7 promoter that is induced upon addition of IPTG) to obtain plasmid pET-T734 (Yoshikawa F. et al., *J. Biol. Chem.* 271:18277-18284, 1996). Using this plasmid (pET-T734) as a parent plasmid, the following expression plasmids were constructed for IP_3-binding polypeptides. Herein, an IP_3-binding polypeptide with high affinity is also referred to as an "IP_3 sponge".

(1-1) Expression plasmid for high affinity IP_3 sponge "T604"

[0077] A gene coding for polypeptide T604 that corresponds to the first methionine (M-1) to the lysine at Position 604 (K-604) of polypeptide T734 was prepared. Specifically, site-directed mutagenesis was conducted by PCR using a complementary oligonucleotide (Yoshikawa F. et al., *J Biol Chem*, 271:18277-18284, 1996) to introduce a stop codon (TAA) and a subsequent *Bam*HI recognition site (GGATCC) at Position 605 of T734.

Sense primer: 5'-TGTCAGACATATGCGTGTTGGAA-3'

*Nde*I (SEQ ID NO: 5)

Antisense primer:

5'-CGCGGGATCCTTATTTCCGGTTGTTGTGGAGCAGGG-3'

*Bam*HI (SEQ ID NO: 6)

[0078] The sense primer was introduced with a *Nde*I cleavage recognition sequence (CATATG) (underlined) including the first methionine codon (ATG).

[0079] A total of 100 µl PCR reaction solution was used. The PCR reaction solution contained 100 ng template DNA, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.2), 2 mM $MgCl_2$, 0.1% TritonX-100, 10 µg/ml BSA, 200 µM dNTPs, 1 µM sense primer, 1 µM anti-sense primer and 2.5 unit Pfu DNA polymerase. The PCR reaction was performed at 95°C for 1 min. and then through 30 cycles of: 95°C for 1 min.; 57°C for 3 min.; and 72°C for 3 min.

[0080] The 5'-end of the obtained amplified fragment was treated with *Nde*I and the 3'-end with *Bam*HI, thereby producing deletion mutant pET-T604 that contains DNA coding for an amino acid sequence corresponding to the amino acid sequence of T734 but with C-terminal deletion up to Position 605.

(1-2) Expression plasmid for high affinity IP_3 sponge "G224"

[0081] First, a gene coding for polypeptide T604 that corresponds to the first methionine (M-1) to the lysine at Position 604 (K-604) of polypeptide T734 was prepared. Specifically, site-directed mutagenesis was conducted by PCR using a complementary oligonucleotide (Yoshikawa F. et al., *J Biol Chem,* 271:18277-18284, 1996) to introduce a stop codon (TAA) and a subsequent *Eco*RI recognition site (GAATCC) at Position 605 of T734.

Sense primer: 5'-TGTCAGACATATGCGTGTTGGAA-3'

*Nde*I (SEQ ID NO: 5)

[0082] Antisense primer:

5'-CCGGAATTCTTATTTCCGGTTGTTGTGGAGCAGGG-3'

*Eco*RI (SEQ ID NO: 7)

[0083] The PCR was conducted under the same conditions as described in (1-1) above.

[0084] The 5'-end of the thus-obtained amplified fragment was treated with *Nde*I and the 3'-end with *Eco*RI, thereby producing deletion mutant pET-T604e that contains DNA coding for an amino acid sequence corresponding to the amino acid sequence of T734 but with C-terminal deletion up to Position 605.

[0085] Then, using deletion mutant pET-T604e as a template, site-directed mutagenesis was performed to introduce *Bam*HI recognition site (GGATCC) immediately before the methionine at Position 224 of polypeptide T604.

[0086] Antisense primer:

5'-CCGGAATTCTTATTTCCGGTTGTTGTGGAGCAGGG-3'

*Eco*RI (SEQ ID NO: 7)

[0087] Sense primer:

5'-CGCGGATCCATGAAATGGAGTGATAACAAAGACGACA-3'

*Bam*HI (SEQ ID NO: 8)

[0088] The PCR was conducted under the same conditions as described in (1-1) above.

[0089] The thus-obtained amplified fragment (plasmid introduced with mutation) was cleaved with *Bam*HI and *Eco*RI,

thereby obtaining a cDNA fragment coding for Amino acids 224-604. This cDNA fragment was ligated to *Bam*HI-*Eco*RI site of GST fusion plasmid (pGEX-2T) without a frameshift (in-frame), thereby obtaining plasmid pGEX-G224. Plasmid pGEX-G224 expresses fusion polypeptide G224 (Fig. 1) that includes polypeptide GST and subsequent polypeptide M-224 to K-604.

(1-3) Expression plasmid for low affinity IP_3-binding polypeptide

[0090] Site-directed mutagenesis was conducted by sequential PCR using pGEX-G224 as a template.

[0091] The following two mismatched oligonucleotides were synthesized to introduce mutation (K508A) at Position 508 of T604 where alanine was substituted for lysine (K-508):

5'-GAGAGCGGCAGGCACTGATGAGGG-3' (SEQ ID NO: 9)

5'-CCCTCATCAGTGCCTGCCGCTCTC-3' (SEQ ID NO: 10)

[0092] Using the above primers, site-directed mutagenesis was conducted by sequential PCR. The PCR conditions and the composition of the reaction solution were as follows:

Primary reaction 1

[0093]

Sense primer: 5'-GAGAGCGGCAGGCACTGATGAGGG-3'

(SEQ ID NO: 9)

[0094] Antisense primer:

5'-CCGGAATTCTTATTTCCGGTTGTTGTGGAGCAGGG-3'

*Eco*RI (SEQ ID NO: 7)

[0095] The PCR was conducted under the same conditions as described in (1-1) above.

Primary reaction 2

[0096] Sense primer:

5'-CGCGGATCCATGAAATGGAGTGATAACAAAGACGACA-3'

*Bam*HI (SEQ ID NO: 8)

[0097] Antisense primer:

5'-CCCTCATCAGTGCCTGCCGCTCTC-3' (SEQ ID NO: 10)

[0098] The PCR was conducted under the same conditions as described in (1-1) above.

Secondary reaction

[0099] Ten µl of the PCR reaction product resulting through Primary reactions 1 and 2, and 1 µM each of primers (SEQ ID NOS: 7 and 8) were used to conduct PCR under the same conditions as the primary reactions.

[0100] The obtained amplified fragment was cleaved with *Bam*HI and *Eco*RI. The cleaved fragment was ligated to *Bam*HI-*Eco*RI site of GST fusion plasmid pGEX-2T without a frameshift (in frame), thereby obtaining plasmid pGEX-G224-m30. This mutant plasmid expresses polypeptide G224-m30 having the point mutation K508A (Fig. 1, m30).

(1-4) Expression plasmid for high affinity IP_3 sponge "G224-m49"

[0101] Using pGEX-G224 as a template, site-directed mutagenesis was conducted by sequential PCR.

[0102] The following two mismatched oligonucleotides were synthesized to introduce a mutation (R441Q) at Position 441 of T604 where glutamine was substituted for alginine (R-441):

5'-GCTGAGGTTCAAGACCTGGACTTTG-3' (SEQ ID NO: 11)

5'-AAAGTCCAGGTCTTGAACCTCAGC-3' (SEQ ID NO: 12)

Primary reaction 1

[0103] Sense primer:

5'-GCTGAGGTTCAAGACCTGGACTTTG-3' (SEQ ID NO: 11)

[0104] Antisense primer:

5'-CCGGAATTCTTATTTCCGGTTGTTGTGGAGCAGGG-3'

*Eco*RI (SEQ ID NO: 7)

[0105] The PCR was conducted under the same conditions as described in (1-3) above.

Primary reaction 2

[0106] Sense primer:

5'-CGCGGATCCATGAAATGGAGTGATAACAAAGACGACA-3'

*Bam*HI (SEQ ID NO: 8)

[0107] Antisense primer:

5'-AAAGTCCAGGTCTTGAACCTCAGC-3' (SEQ ID NO: 12)

[0108] The PCR was conducted under the same conditions as described in (1-3) above.

Secondary reaction

[0109] Ten µl of the PCR reaction product resulting through Primary reactions 1 and 2, and 1 µM each of primers (SEQ ID NOS: 6 and 8) were used to conduct PCR under the same conditions as those of the primary reactions.

[0110] The obtained amplified fragment was cleaved at a *Bam*HI-*Eco*RI site. The cleaved fragment was ligated to *Bam*HI-*Eco*RI site of GST fusion plasmid pGEX-2T without a frameshift (in frame), thereby obtaining plasmid pGEX-G224-m49. This mutant plasmid expresses polypeptide G224-m49 having the point mutation R441Q (Fig. 1, m49).

Example 2: Expression and Preparation of

High Affinity IP_3-Binding Polypeptide with *E.coli*

[0111] Since the IP_3-binding core mostly results in insoluble inclusion bodies, the expression amount is low. Thus, the present inventors have modified the IP_3-binding region through gene engineering to produce a high affinity IP_3-binding polypeptide which is of lower molecule, which is capable of stable mass-expression, which can be recovered as a soluble protein, which has a higher affinity, and which has as high specificity as a conventional IP_3 receptor.

[0112] By low-temperature cultivation (16-22°C), polypeptide T734 can be mass-expressed in a stable manner with a relatively high soluble fraction recovery (Kd = 50 ± 2.4 nM, B_{max} = 46 pmol/mg protein, 1.85 mg/l *E.coli* culture (corresponding to about 0.5 g of wet *E.coli*)). However, the inclusion bodies amount to more than ten times the amount of the soluble fraction (Yoshikawa F. et al., *J. Biol Chem.* 271: 18277-18284, 1996).

[0113] First of all, smaller polypeptides that had the above-described characteristics were prepared.

[0114] The pET-type and pGEX-type expression plasmids obtained in Example 1 were introduced into *E.coli* BL21 (DE3) and JM109, respectively, by transformation method. Expression induction with IPTG and preparation of expression proteins from *E.coli* were mainly conducted by modifying the method of Yoshikawa et al (Yoshikawa F. et al., *J. Biol Chem.* 271: 18277-18284, 1996).

[0115] Specifically, *E.coli* introduced with respective plasmids were shake cultured in L broths (containing 100 µg/ml ampicillin) at 22°C. When the absorption OD_{600} became about 1.5, IPTG was added to 0.5 mM. After a few hours of shake culture at 16°C, each of the *E.coli* was recovered through centrifugation and suspended in PBS containing protease inhibitors (1 mM PMSF, 10 µM leupeptin, 1 µM pepstatin A, 2 µg/ml aprotinin). Each of the *E.coli* was disrupted by sonication. Then, each supernatant containing the expression polypeptide (soluble fraction) was collected by ultracentrifugation (Beckman Ti35 rotor, 25,000 rpm, 1 hr., 4°C).

[0116] GST fusion polypeptides were purified from the soluble fractions by affinity purification using Glutathione-Sepharose column (Pharmacia LKB). Specifically, each of the GST fusion polypeptides was eluted from the column with 10 mM glutathione/50 mM Tris-HCl (pH 8.0) by mainly following the manual provided by the manufacturer. The polypeptide solutions were equilibrated with 10 mM HEPES-KOH (pH 7.2), 88 mM NaCl and 1 mM KCl using PD10 desalted column (Pharmacia LKB), and then dispensed, thereby obtaining the IP_3 sponges (Fig. 1: G224, m30, m49 and GST). The IP_3 sponges were stored at -80°C until they were used.

[0117] A series of deletion mutants based on polypeptide T734 were prepared by serially shortening the length of the polypeptide T734 from the C-terminus. The analysis of the deletion mutants indicated that T705 and T699 had no marked characteristic difference with T734. In the cases of polypeptides T569, T572 and T576, the expression amounts of the soluble proteins were lower than T734. Stable mass-expression of soluble protein was successful with polypeptide T604 which was obtained by deleting the C-terminus of T734 up to Amino acid 605 (Fig. 1).

[0118] With reference to Fig. 1, the uppermost (IP_3R1) is the N-terminal amino acids of the IP_3 receptor including the IP_3-binding core region (core: Amino acids 226-578). T604 (Amino acids 1-604), G224 (GST + Amino acids 224-604), G224m30 (G224 introduced with K508A mutation), G224m49 (G224 introduced with R441Q mutation), and GST (derived from pGEX-2T)) are also shown in Fig. 1.

[0119] T604 had a [^{3}H]IP_3-binding activity substantially equivalent to that of T734 (Kd = 45 nM), and a higher yield of soluble protein (B_{max} = 690 pmol/mg protein). Specifically, the yield was 19 mg/l *E.coli* culture (Figs. 2B and 2C, Table 1).

Table 1

Expression of IP_3-binding site in *E.coli*			
Protein	Expression efficiency (mg/l *E.coli* culture)	Kd [nM]	B_{max} [pmol/µg purified protein]
Purified IP_3R^a	-	83	2.1

a. Maeda et al., *EMBO J.* 9, 51-67, 1990

Table 1 (continued)

Expression of IP_3-binding site in *E.coli*			
Protein	Expression efficiency (mg/l *E.coli* culture)	Kd [nM]	B_{max} [pmol/μg purified protein]
T734[b]	1.85	(50)[c]	ND
T604	19	7.6/(45)c	ND
G224	30	0.083	1.6
G224m49	ND	0.043	1.7
G224m30	ND	330	3.0

b. Yoshikawa et al., *J. Biol. Chem.*, 271, 18277-18284, 1996

c. the values in parentheses represent Kd obtained from crude cell lysates

ND. Not Determined

[0120] The total expression amount of polypeptide T604 substantially equaled to that of polypeptide T734 but T604 had a remarkably improved soluble protein yield. The yield of soluble protein of polypeptide T604 was substantially the same at 30°C and 37°C, and reached the peak within 2 hours after initiating expression induction.

[0121] Fig. 2A shows the result of Western blotting analysis of the protein (0.1 μg) obtained from an *E.coli* extract solution (soluble fraction) that expresses polypeptide T604 (66kDa). As a control, a cell extract solution obtained by transforming a vector that does not include T604 (pET-3a) was used. Fig. 2B shows a comparison of the total amounts of specific IP_3-binding contained in 0.7 μl soluble fractions, for T734, T604, and the control vector. Fig. 2C shows the result of Schatchard plot analysis where the binding between 3 μg of T604 soluble fraction and 9.6 nM [^{3}H]IP_3 was competitively inhibited with non-labeled IP_3 (cold IP_3) at various concentrations. The results were Kd = 45 ± 7.6, B_{max} = 690 ± 64 pmol/mg protein.

[0122] When T734 was serially deleted from the N-terminus, a very short N-terminal deletion of T734 (e.g., a deletion of 31 amino acids) caused lack of IP_3-binding activity even the deletion was outside the core region. However, the polypeptide retrieved the IP_3-binding activity when the N-terminus was deleted to Amino acid 220-225, near the N-terminus of the core region (Yoshikawa et al, 1996). The theory for this is unknown, but presumably, the formation of the three-dimensional structure for active core region is somehow interrupted depending on the degree of deletion. Although the active polypeptide with the N-terminal deletion up to Amino acid 220-225 had a relatively high affinity, the amount of soluble protein expressed was lower.

[0123] As described above, a protein obtained by deleting Amino acids 1-223 of polypeptide T604 (N4-T604; Amino acids 224-604) had a higher activity (about 3 times high) but lower production than those of the original T604. Accordingly, polypeptide T604 seemed to be the most suitable polypeptide for stably mass-expressing only the high affinity IP_3-binding region as a soluble protein.

Example 3: Expression of IP_3 Sponge

(i) [^{3}H] -IP_3-binding inhibition experiment

[0124] Based on the results obtained in Example 2, an IP_3-binding polypeptide with a higher affinity was produced. As described above, when the amino-terminal Amino acids 1-223 of polypeptides T604 and T734 were deleted, high [^{3}H]IP_3-binding activities were obtained. Even Amino acid region 224-579 (a polypeptide that almost corresponds to the core region) consisting of only 356 amino acid residues has an affinity as high as Kd = 2.3 nM (Yoshikawa et al., 1996, *supra*). However, as described above, these polypeptides have lower soluble protein expression levels. In other words, longer amino terminal deletion may result in a higher affinity on one hand, but it also lowers the expression amount and expression stability of soluble proteins by rendering most of proteins as insoluble inclusion bodies.

[0125] In general, stability, solubility and an expression level of a foreign polypeptide are known to be improved when it is made into a GST fusion body. In this example, fusion proteins G224, G224-m30 and G224-m49 consisting of GST and an IP_3-binding site (Amino acid region 224-604) were prepared by ligating GST to replace the N-terminal region (Amino acids 1-223) of the IP_3 receptor (Fig. 1).

[0126] The IP_3-binding activities of these fusion proteins were measured mainly by the method of Yoshikawa et al (1996).

[0127] Each fusion protein (IP_3 sponge) (0.2 μg) was mixed with 100 μl of binding buffer-α (50 mM Tris-HCl (pH 8.0 at 4°C), 1 mM EDTA, 1 mM β-mercaptoethanol) that contained 9.6 nM D-myo-[^{3}H](1,4,5)IP_3 (777 GBq/mmol; DuPont NEN) (hereinafter, abbreviated as "[^{3}H]IP_3") and various concentrations of non-labeled D-myo-(1,4,5)IP_3 (Dojindo) (hereinafter, abbreviated as "cold IP_3"). The mixture was left to stand on ice for 10 minutes. To the mixture, 4 μl of 50

mg/ml γ-globulin (Sigma) (final concentration: 1 mg/ml) and 100 µl of 30% PEG 6000 (Sigma)/binding buffer-α solution (final concentration: 15%) were added. The resultant mixture was left to stand on ice for 5 minutes, and then centrifuged at 10,000 x g at 2°C for 5 minutes to collect polypeptide/PEG complex. PEG-precipitated [^{3}H]IP_3-binding polypeptide was well solubilized with 180 µl solubilizer Solvable (DuPont NEN) . The resultant was neutralized with 18 µl glacial acetic acid and then added to 5 ml liquid scintillation counter (Atomlight [DuPont NEN]) to measure the radioactivity (first radioactivity) . Nonspecific binding of each protein was determined by measuring the second radioactivity in the presence of 2 µM or 10 µM cold IP_3. Then, a specific binding value of each protein was obtained by subtracting the second radioactivity (nonspecific binding value) from the first radioactivity values.

[0128] Scatchard plot analysis was conducted under the following conditions. For low-affinity polypetides (G224-m30 and control GST), the binding experiment was conducted in 100 µl binding buffer α by adding 9.6 nM [^{3}H]IP_3 (DuPont NEN) and 10-20 nM of cold IP_3 to 2 µg of IP_3-binding polypeptide, and by adding 9.6 nM [^{3}H]IP_3 (DuPont NEN) and 50 nM-2 µM of cold IP_3 to 0.01 µg IP_3-binding polypeptide. For high-affinity IP_3 sponges (G224 and G224-m49), binding experiment was conducted with 0.02 µg IP_3 sponges at [^{3}H]-IP_3 concentrations of 0.15, 0.3, 0.6, 1.2, 2.4, 4.8 and 9.6 nM without adding cold IP_3.

[0129] The inhibition effects of the IP_3-binding polypeptides (IP_3 sponges) on [^{3}H]IP_3-binding activity of cerebellar microsome was analyzed as follows.

[0130] A microsomal fraction was prepared from the cerebella of mice *ddY* (Nippon SLC) mainly by following the method of Nakada et al. (Nakada S. et al., *Biochem. J.* 277:125-131, 1991). In 100 µl of binding buffer α, various concentrations of the IP_3 sponges were added respectively to see the changes in the binding between the cerebellar microsome (40 µg) and 9.6 nM [^{3}H]-IP_3 according to the above method (see Scatchard plot analysis).

[0131] As a result, the affinity of polypeptide G224 was found out to be 500 times higher than that of polypeptide T734 (Kd = 83 pM, B_{max} = 1.6 pmol/µg protein) (Fig. 3A). Polypeptide G224 binds well to (1,4,5)IP_3 and (2,4,5)IP_3 and the yield of IP_3-binding protein was about 30 mg/l *E.coli* culture (Table 1). After purifying the protein with a glutathione column and a subsequent PD10 column, the yield was about 24 mg/l. The binding activity was augmented when R441Q mutation was introduced into polypeptide T734 (Yoshikawa et al., 1996, *supra*). The affinity of polypeptide G224-m49 (G224 introduced with R441Q mutation) doubled and became about 1,000 times higher than that of polypeptide T734 (Kd = about 43 pM, B_{max} = 1.7 pmol/µg protein) (Fig. 3B, Table 1). The binding activity decreased when polypeptide T734 was introduced with K508A mutation (Yoshikawa et al., 1996 (*supra*)). Similarly, the binding activity of polypeptide G224-m30 decreased when G224 was introduced with K508A mutation and became as low as about 1/4,000 of polypeptide G224 and about 1/7,700 of polypeptide G224-m49 (Kd = about 330 nM, B_{max} = 3.0 pmol/µg protein) (Fig. 3C, Table 1).

(ii) IP_3-binding inhibition via absorption by novel IP_3 sponge

[0132] IP_3-binding polypeptides G224 and G224-m49 have powerful IP_3-binding activities that are 500 to 1,000 times higher than that of the original IP_3 receptor. Polypeptides G224 and G224-m49 were tested for their use as an IP_3-specific absorption body (sponge) (IP_3 sponge), i.e., whether they can decrease the amount of IP_3-binding by the IP_3 receptors in a solution by competitively absorbing IP_3 in the solution (Fig. 4).

[0133] Mouse cerebellum is a tissue that is rich in IP_3 receptor and whose microsomal fraction has a [^{3}H]IP_3-binding activity which is at least 50 times higher than those in other tissues (Maeda et al., 1990 (*supra*)). Binding between 40 µg cerebellar microsome (Kd = 21 nM, B_{max} = 23 pmol/mg protein) and 9.6 nM [^{3}H]IP_3 in 100 µl solution was analyzed for percentage (%) of competitive inhibition at various concentrations of IP_3 sponges where the activity under the absence of IP_3 sponge was considered 100%. It was calculated that, there were about 0.92 pmol of IP_3-binding site of cerebellum IP_3 receptor and 0.96 pmol of [^{3}H]IP_3 present in the 100 µl solution.

[0134] As a result, no inhibition effect was observed for control GST even when the IP_3 sponge concentration was 100 pg/ml (Fig. 4) . On the other hand, for high-affinity polypeptides G224 and G224-m49, strong IP_3-binding inhibition activities were observed and IC_{50} was about 10 µg/ml (Fig. 4). Polypeptide G224-m30 with low affinity had low inhibition activity with IC_{50} of 100 µg/ml. According to this *in vitro* experiment system, the IP_3 sponge tended to precipitate with the microsome membrane when the IP_3 sponge concentration exceeded about 25 µg/ml, and so the concentration-dependent curves were likely to fluctuate (Fig. 4). Thus, the apparent inhibition of G224-m30 observed at IP_3 sponge concentration exceeding 25 µg/ml could be due to precipitation under high concentration.

[0135] These results show that [^{3}H]IP_3-binding of the IP_3 receptor can efficiently be inhibited according to the binding affinity and the concentration of the IP_3 sponge used. High affinity IP_3-binding polypeptide of the invention is a novel IP_3 sponge that can be used as an IP_3 neutralizing agent, or an antagonist for IP_3-induced calcium.

Example 4: Test of Inhibiting IP_3-Induced Ca^{2+} Release (IICR)

[0136] To conduct a test of inhibiting IP_3-induced Ca^{2+} release, a microsomal fraction was prepared from mouse

cerebellum as described in Example 3. The fraction was suspended in Buffer B, dispensed, and stored at -80°C until it was used.

[0137] Composition of Buffer B was 110 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4, 1 mM DDT, and 50 mM HEPES-KOH (pH 7.2) (containing a cocktail of protease inhibitors [0.1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A, 10 μM E-64] and 2 mM $MgCl_2$).

[0138] An IP_3-induced Ca^{2+} release activity of cerebellar microsome was determined by using fura-2 (Molecular Probe) as a fluorescent Ca^{2+} indicator. Specifically, excitations upon addition of IP_3 at two wavelengths (340 nm and 380 nm) were measured with fluorescence spectrophotometer CAF110 (Nihon Bunko) to see the change in the fluorescent intensity ratio (F340/F380) at 500 nm.

[0139] IP_3-induced Ca^{2+} release from the cerebellar microsome is generally EC_{50} = 100-200 nM IP_3. Cerebellar microsome (100 μg) was mixed with 500 μl of a release buffer (Buffer B containing 1 mM $MgCl_2$, 2 μM fura-2, 1 mM DTT, 10 mM creatine phosphate, 40U/ml creatine kinase, 1 μg/ml oligomycin, and the cocktail of protease inhibitors) in a measurement cuvette with a stirrer bar. The following reaction was conducted at 30°C while constantly stirring with the stirrer bar.

[0140] One mM of ATP was added to the mixture in the cuvette to activate Ca^{2+} pumping (Ca^{2+}-ATPase), whereby Ca^{2+} was incorporated into the inner space of microsome (Ca^{2+} loading). Ca^{2+} loading was confirmed by monitoring until the decrease of fura-2 fluorescent level became constant. The change in the fura-2 fluorescent intensity ratio was measured (F340/F380) at a subthreshold level.

[0141] The effect of IP_3 sponge on inhibiting IP_3-induced Ca^{2+} release activity of cerebellar microsome was analyzed as follows. After the addition of ATP, the curve of fura-2 fluorescent intensity was monitored until the decrease became constant. Then, various concentrations of IP_3 sponges were added. After 1 min., 50 nM to 1 μM of IP_3 was added to the reaction mixture to observe the change of fura-2 fluorescent intensity induced by the IP_3.

[0142] The IP_3 sponge concentration dependency was determined as follows. High affinity polypeptide G224 of 3.125, 6.25, 12.5, 25, 50, 100, 200 μg/ml were added to the reaction mixture, respectively. After about 1 min., 100 nM of IP_3 was added to measure the Ca^{2+} release activity induced by the IP_3. The concentration dependency of low affinity polypeptide G224-m30 was determined by adding G224-m30 of 200, 400 and 500 μg/ml. After about 1 min., 100 nM of IP_3 was added to measure the Ca^{2+} release activity induced by the IP_3. In addition, G224-m30 of 500 μg/ml was also added, and after about 1 min., 50 nM of IP_3 was added to measure the Ca^{2+} release activity induced by the IP_3.

[0143] As a result, it was found that the IP_3 sponges specifically inhibited in a competitive manner the IP_3-binding by the IP_3 receptor of cerebellar microsome by absorbing the IP_3 (Figs. 5A-5F, 6A-6G and 7). In Figs. 5A-5F and 6A-6G, the vertical axis represent the change in fura-2 fluorescent intensity ratio (F340/F380) (i.e., change in the amount of Ca^{2+}), and the horizontal axis represents the time (sec).

[0144] As shown in Figs. 5A, 5B and 5C, in the absence of IP_3 sponge (controls), IP_3-induced Ca^{2+} release activities were dependent on IP_3 concentration. Low-affinity polypeptide G224-m30 at a concentration of 500 μg/ml had no inhibiting effect on Ca^{2+} release with 100 nM IP_3 (Fig. 5E). Little difference was found between G224-m30 and the control for effects on inhibiting 50 nM IP_3 (Fig. 5E). With GST only, even at a high concentration of 632 μg/ml, no change was seen in Ca^{2+} release activity induced with 50 nM IP_3 (Fig. 5F). Thus, in each case, no marked difference was noted with the control.

[0145] On the contrary to the above results, high affinity polypeptide G224 had a significant inhibition effect on IP_3-induced Ca^{2+} release depending on its concentration (Figs. 6A-6G). The high affinity polypeptide G224 had the greatest inhibition effect at 100 μg/ml and almost completely inhibited the IP_3-induced Ca^{2+} release (Fig. 6F).

[0146] The peak values of Ca^{2+} release obtained by adding G224 at each concentration shown in Figs. 6A-6G, were plotted where the peak obtained in the absence of G224 was considered 100 % (Fig. 7). The horizontal axis represents each concentration of polypeptide G224 and the verticle axis represents the peak value of Ca^{2+} release. As can be appreciated from Fig. 7, the concentration of polypeptide G224 required for 50 % inhibition of IP_3-induced Ca^{2+} release was about 20 μg/ml.

[0147] Accordingly, it was found that the high affinity IP_3-binding polypeptide acted as an IP_3 sponge and specifically inhibited, in a concentration-dependant manner, the IP_3-induced Ca^{2+} release by the IP_3 receptor on cerebellar microsome.

[0148] The present invention provides a polypeptide having a high affinity binding activity to inositol 1,4,5-trisphosphate, a gene encoding the polypeptide, a recombinant vector including the gene, a transformant including the vector and a method for producing the polypeptide.

[0149] The polypeptide of the invention can be used to control the inhibition of a specific cell function that depends on an IP_3-induced calcium signal transmission (IP_3 neutralizing agent, antagonist for IP_3-induced calcium, etc.). Furthermore, the polypeptide and the gene of the present invention is useful as an IP_3 signal detecting agent for inhibiting activation of IP_3-induced calcium signal transmission. The gene of the invention is also useful as a therapeutic agent for treating a disease associated with calcium production.

[0150] Various other modifications will be apparent to and can be readily made by those skilled in the art without

departing from the scope and spirit of this invention. Accordingly, it is not intended that the scope of the claims appended hereto be limited to the description as set forth herein, but rather that the claims be broadly construed.

[0151] All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

[0152] The following are information on sequences described herein:

Annex to the description

[0153]

SEQUENCE LISTING

<110> RIKEN ; Katsuhiko Mikoshiba

<120> High affinity IP3 binding polypeptide

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Lys Ile Leu Leu Ala Ile Leu Asp Cys Val His Val Thr Thr Ile Phe

890 895 900

ccc att agc aag atg aca aaa gga gaa gag aat aaa ggc agt aac gtg 3088
Pro Ile Ser Lys Met Thr Lys Gly Glu Glu Asn Lys Gly Ser Asn Val

905 910 915 920

atg agg tct atc cat ggc gtt ggg gag ctg atg acc cag gtg gtg ctg 3136

Met Arg Ser Ile His Gly Val Gly Glu Leu Met Thr Gln Val Val Leu
925 930 935

cgg gga gga ggc ttc ttg ccc atg act ccc atg gct gcg gcc cct gaa 3184
Arg Gly Gly Gly Phe Leu Pro Met Thr Pro Met Ala Ala Ala Pro Glu
940 945 950

gga aat gtg aag cag gca gag cca gag aaa gag gac atc atg gtc atg 3232
Gly Asn Val Lys Gln Ala Glu Pro Glu Lys Glu Asp Ile Met Val Met
955 960 965

gac acc aag ttg aag atc att gaa ata ctc cag ttt att ttg aat gtg 3280
Asp Thr Lys Leu Lys Ile Ile Glu Ile Leu Gln Phe Ile Leu Asn Val
970 975 980

aga ttg gat tat agg atc tcc tgc ctc ctg tgt ata ttt aag cga gag 3328
Arg Leu Asp Tyr Arg Ile Ser Cys Leu Leu Cys Ile Phe Lys Arg Glu
985 990 995 1000

ttt gat gaa agc aat tcc cag tca tca gaa aca tcc tcc gga aac agc 3376
Phe Asp Glu Ser Asn Ser Gln Ser Ser Glu Thr Ser Ser Gly Asn Ser
1005 1010 1015

agc cag gaa ggg cca agt aat gtg cca ggt gct ctt gac ttt gaa cac 3424
Ser Gln Glu Gly Pro Ser Asn Val Pro Gly Ala Leu Asp Phe Glu His
1020 1025 1030

att gaa gaa caa gcg gaa ggc atc ttt gga gga agt gag gag aac aca 3472
Ile Glu Glu Gln Ala Glu Gly Ile Phe Gly Gly Ser Glu Glu Asn Thr
1035 1040 1045

cct ttg gac ctg gat gac cat ggt ggc aga acc ttc ctc agg gtc ctg 3520
Pro Leu Asp Leu Asp Asp His Gly Gly Arg Thr Phe Leu Arg Val Leu
1050 1055 1060

ctc cac ttg aca atg cat gac tac cca ccc ctg gtg tct ggg gcc ctg 3568
Leu His Leu Thr Met His Asp Tyr Pro Pro Leu Val Ser Gly Ala Leu
1065 1070 1075 1080

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cag ctc ctc ttt cgg cac ttc agc cag agg cag gag gtc ctt cag gcc   3616
Gln Leu Leu Phe Arg His Phe Ser Gln Arg Gln Glu Val Leu Gln Ala
                1085                1090                1095

ttc aaa cag gtt caa ctg ctg gtt act agc caa gat gtg gac aac tac   3664
Phe Lys Gln Val Gln Leu Leu Val Thr Ser Gln Asp Val Asp Asn Tyr
            1100                1105                1110

aaa cag atc aag caa gac ttg gac caa cta agg tcc att gtg gag aag   3712
Lys Gln Ile Lys Gln Asp Leu Asp Gln Leu Arg Ser Ile Val Glu Lys
        1115                1120                1125

tct gag ctc tgg gtg tac aaa ggc caa ggt ccc gat gag cct atg gac   3760
Ser Glu Leu Trp Val Tyr Lys Gly Gln Gly Pro Asp Glu Pro Met Asp
    1130                1135                1140

gga gcc tcc ggt gaa aat gag cat aag aaa acc gag gag ggg acg agc   3808
Gly Ala Ser Gly Glu Asn Glu His Lys Lys Thr Glu Glu Gly Thr Ser
1145                1150                1155                1160

aag cca ctg aag cac gag agc acc agc agc tac aac tac cga gtg gtg   3856
Lys Pro Leu Lys His Glu Ser Thr Ser Ser Tyr Asn Tyr Arg Val Val
                1165                1170                1175

aaa gag att ttg att cga ctt agc aag ctc tgc gtg cag gag agc gcg   3904
Lys Glu Ile Leu Ile Arg Leu Ser Lys Leu Cys Val Gln Glu Ser Ala
            1180                1185                1190

tcg gtg agg aag agc cgg aag cag cag caa cga ctg ctg agg aac atg   3952
Ser Val Arg Lys Ser Arg Lys Gln Gln Gln Arg Leu Leu Arg Asn Met
        1195                1200                1205

ggc gca cac gct gtg gtg ctg gag ctg ctg cag atc ccc tac gag aag   4000
Gly Ala His Ala Val Val Leu Glu Leu Leu Gln Ile Pro Tyr Glu Lys
    1210                1215                1220

gcc gaa gac aca aag atg caa gag atc atg cgg ctg gct cat gaa ttt   4048
Ala Glu Asp Thr Lys Met Gln Glu Ile Met Arg Leu Ala His Glu Phe
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1225 1230 1235 1240

ttg cag aat ttc tgt gca ggc aac cag cag aat caa gct ttg ctg cat 4096

Leu Gln Asn Phe Cys Ala Gly Asn Gln Gln Asn Gln Ala Leu Leu His

1245 1250 1255

aaa cac ata aac ctg ttt ctc aag cca ggg atc ctg gag gca gtg acg 4144

Lys His Ile Asn Leu Phe Leu Lys Pro Gly Ile Leu Glu Ala Val Thr

1260 1265 1270

atg cag cac atc ttc atg aac aac ttc cag ctg tgc agt gag atc aac 4192

Met Gln His Ile Phe Met Asn Asn Phe Gln Leu Cys Ser Glu Ile Asn

1275 1280 1285

gag aga gtg gtc cag cac ttt gtt cac tgc ata gag acc cac ggt cga 4240

Glu Arg Val Val Gln His Phe Val His Cys Ile Glu Thr His Gly Arg

1290 1295 1300

aac gtc cag tat atc aag ttt ctc cag acg att gtc aag gca gaa ggg 4288

Asn Val Gln Tyr Ile Lys Phe Leu Gln Thr Ile Val Lys Ala Glu Gly

1305 1310 1315 1320

aaa ttc att aaa aag tgc caa gac atg gtc atg gct gag ctt gtc aac 4336

Lys Phe Ile Lys Lys Cys Gln Asp Met Val Met Ala Glu Leu Val Asn

1325 1330 1335

tct gga gag gac gtc ctc gtg ttc tac aat gac aga gcc tct ttc cag 4384

Ser Gly Glu Asp Val Leu Val Phe Tyr Asn Asp Arg Ala Ser Phe Gln

1340 1345 1350

act ctg atc cag atg atg cgg tcc gag cgt gac cgg atg gat gag aac 4432

Thr Leu Ile Gln Met Met Arg Ser Glu Arg Asp Arg Met Asp Glu Asn

1355 1360 1365

agc cct ctc atg tac cac atc cat ctg gtg gag ctc ttg gcc gtg tgc 4480

Ser Pro Leu Met Tyr His Ile His Leu Val Glu Leu Leu Ala Val Cys

1370 1375 1380

aca gag ggc aag aat gtg tac acg gag atc aag tgc aac tcc ttg ctc 4528

Thr Glu Gly Lys Asn Val Tyr Thr Glu Ile Lys Cys Asn Ser Leu Leu

1385 1390 1395 1400

ccg ctc gat gac atc gtt cgt gtg gtc act cat gaa gac tgc atc ccc 4576

Pro Leu Asp Asp Ile Val Arg Val Val Thr His Glu Asp Cys Ile Pro

1405 1410 1415

gag gtt aag atc gct tac att aac ttc ctg aat cac tgc tat gtg gat 4624

Glu Val Lys Ile Ala Tyr Ile Asn Phe Leu Asn His Cys Tyr Val Asp

1420 1425 1430

acg gag gtg gag atg aag gag att tac aca agc aac cac atg tgg aag 4672

Thr Glu Val Glu Met Lys Glu Ile Tyr Thr Ser Asn His Met Trp Lys

1435 1440 1445

ttg ttt gag aat ttc ctc gtg gac atc tgc agg gcc tgt aac aac aca 4720

Leu Phe Glu Asn Phe Leu Val Asp Ile Cys Arg Ala Cys Asn Asn Thr

1450 1455 1460

agc gac agg aag cac gca gac tcc att ctg gag aag tac gtc act gaa 4768

Ser Asp Arg Lys His Ala Asp Ser Ile Leu Glu Lys Tyr Val Thr Glu

1465 1470 1475 1480

atc gtg atg agc atc gtc acc acc ttc ttc agc tct ccc ttc tca gac 4816

Ile Val Met Ser Ile Val Thr Thr Phe Phe Ser Ser Pro Phe Ser Asp

1485 1490 1495

cag agc acc act ctg cag acc cgc cag cct gtc ttt gtg caa ctc ctg 4864

Gln Ser Thr Thr Leu Gln Thr Arg Gln Pro Val Phe Val Gln Leu Leu

1500 1505 1510

caa ggc gtg ttc cga gtt tac cac tgc aac tgg ctg atg ccg agc caa 4912

Gln Gly Val Phe Arg Val Tyr His Cys Asn Trp Leu Met Pro Ser Gln

1515 1520 1525

aaa gcc tcg gtg gag agc tgc atc cgg gtg ctc tct gac gta gcc aag 4960

Lys Ala Ser Val Glu Ser Cys Ile Arg Val Leu Ser Asp Val Ala Lys

1530 1535 1540

agc cgg gcc ata gcc att cct gtt gac ctg gac agc caa gtc aac aac 5008
Ser Arg Ala Ile Ala Ile Pro Val Asp Leu Asp Ser Gln Val Asn Asn
1545 1550 1555 1560

ctc ttc ctg aag tcc cac aac att gtg cag aaa aca gcc ctg aac tgg 5056
Leu Phe Leu Lys Ser His Asn Ile Val Gln Lys Thr Ala Leu Asn Trp
1565 1570 1575

cgg tta tca gcc cga aac gcc gct cgc aga gac tct gta ctg gca gca 5104
Arg Leu Ser Ala Arg Asn Ala Ala Arg Arg Asp Ser Val Leu Ala Ala
1580 1585 1590

tcc aga gac tac cga aat atc att gag agg tta cag gac atc gtg tct 5152
Ser Arg Asp Tyr Arg Asn Ile Ile Glu Arg Leu Gln Asp Ile Val Ser
1595 1600 1605

gcc cta gag gac cgg ctc agg ccc ctg gtg cag gct gag ctg tct gtg 5200
Ala Leu Glu Asp Arg Leu Arg Pro Leu Val Gln Ala Glu Leu Ser Val
1610 1615 1620

ctc gtg gat gtt cta cac aga cca gaa ctg ctc ttc ccc gag aac acg 5248
Leu Val Asp Val Leu His Arg Pro Glu Leu Leu Phe Pro Glu Asn Thr
1625 1630 1635 1640

gat gcc agg agg aaa tgt gag agt gga ggt ttc atc tgc aag cta ata 5296
Asp Ala Arg Arg Lys Cys Glu Ser Gly Gly Phe Ile Cys Lys Leu Ile
1645 1650 1655

aaa cat acc aag caa ctg ctg gag gag aat gaa gag aaa cta tgc att 5344
Lys His Thr Lys Gln Leu Leu Glu Glu Asn Glu Glu Lys Leu Cys Ile
1660 1665 1670

aaa gtc tta cag acc ctc agg gaa atg atg acc aaa gac aga ggc tat 5392
Lys Val Leu Gln Thr Leu Arg Glu Met Met Thr Lys Asp Arg Gly Tyr
1675 1680 1685

gga gag aag caa att tcc att gat gaa tcg gaa aat gcc gag ctg cca 5440
Gly Glu Lys Gln Ile Ser Ile Asp Glu Ser Glu Asn Ala Glu Leu Pro

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    1690                1695                1700

cag gca ccg gaa gct gag aac tcc aca gag cag gag ctt gaa cca agt   5488
Gln Ala Pro Glu Ala Glu Asn Ser Thr Glu Gln Glu Leu Glu Pro Ser
1705                1710                1715                1720

cca ccc ctg agg caa ctg gaa gac cat aaa agg ggt gag gca ctc cga   5536
Pro Pro Leu Arg Gln Leu Glu Asp His Lys Arg Gly Glu Ala Leu Arg
                1725                1730                1735

caa att ttg gtc aac cgt tac tat gga aac atc aga cct tca gga aga   5584
Gln Ile Leu Val Asn Arg Tyr Tyr Gly Asn Ile Arg Pro Ser Gly Arg
            1740                1745                1750

aga gag agc ctt acc agc ttt ggc aat ggc cca cta tca cca gga gga   5632
Arg Glu Ser Leu Thr Ser Phe Gly Asn Gly Pro Leu Ser Pro Gly Gly
        1755                1760                1765

ccc agc aag cct ggt gga gga ggg gga ggt cct gga tct agt tcc aca   5680
Pro Ser Lys Pro Gly Gly Gly Gly Gly Gly Pro Gly Ser Ser Ser Thr
    1770                1775                1780

agc agg ggt gag atg agc ctg gct gag gtt cag tgt cac ctc gac aag   5728
Ser Arg Gly Glu Met Ser Leu Ala Glu Val Gln Cys His Leu Asp Lys
1785                1790                1795                1800

gag ggg gcc tcc aac ctg gtc atc gat ctc ata atg aat gca tcc agt   5776
Glu Gly Ala Ser Asn Leu Val Ile Asp Leu Ile Met Asn Ala Ser Ser
                1805                1810                1815

gac cga gta ttc cat gaa agc att ctg ctg gcc atc gca ctt ctg gaa   5824
Asp Arg Val Phe His Glu Ser Ile Leu Leu Ala Ile Ala Leu Leu Glu
            1820                1825                1830

gga ggc aac acc acc atc cag cac tcg ttt ttc tgc cgg ctg aca gaa   5872
Gly Gly Asn Thr Thr Ile Gln His Ser Phe Phe Cys Arg Leu Thr Glu
        1835                1840                1845

gat aag aaa tca gag aag ttc ttc aag gtt ttt tac gat cga atg aag   5920
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Asp Lys Lys Ser Glu Lys Phe Phe Lys Val Phe Tyr Asp Arg Met Lys
  1850                1855                1860

gtg gcc cag cag gaa atc aag gcg aca gtg aca gtg aac acc agc gac   5968
Val Ala Gln Gln Glu Ile Lys Ala Thr Val Thr Val Asn Thr Ser Asp
1865                1870                1875                1880

ttg gga aac aaa aag aaa gat gat gaa gtg gac agg gat gcc ccg tct   6016
Leu Gly Asn Lys Lys Lys Asp Asp Glu Val Asp Arg Asp Ala Pro Ser
                1885                1890                1895

cgg aag aaa gcc aaa gag ccc aca aca cag ata aca gaa gag gtc cgg   6064
Arg Lys Lys Ala Lys Glu Pro Thr Thr Gln Ile Thr Glu Glu Val Arg
            1900                1905                1910

gat cag ctc ctg gaa gca tct gct gcc acc agg aaa gcc ttt acc acc   6112
Asp Gln Leu Leu Glu Ala Ser Ala Ala Thr Arg Lys Ala Phe Thr Thr
        1915                1920                1925

ttc cgg agg gag gcc gac cct gat gac cat tac cag tct ggg gag ggc   6160
Phe Arg Arg Glu Ala Asp Pro Asp Asp His Tyr Gln Ser Gly Glu Gly
    1930                1935                1940

acc cag gct aca acc gac aaa gcc aag gat gac cta gag atg agc gct   6208
Thr Gln Ala Thr Thr Asp Lys Ala Lys Asp Asp Leu Glu Met Ser Ala
1945                1950                1955                1960

gtc atc acc atc atg cag cct atc ctg cgc ttc ctg cag ctg ctg tgt   6256
Val Ile Thr Ile Met Gln Pro Ile Leu Arg Phe Leu Gln Leu Leu Cys
                1965                1970                1975

gaa aac cac aac cga gat ctg cag aat ttc ctt cgt tgc caa aat aat   6304
Glu Asn His Asn Arg Asp Leu Gln Asn Phe Leu Arg Cys Gln Asn Asn
            1980                1985                1990

aag acc aac tac aat ttg gtg tgt gag aca ctg cag ttt ctg gac tgt   6352
Lys Thr Asn Tyr Asn Leu Val Cys Glu Thr Leu Gln Phe Leu Asp Cys
        1995                2000                2005
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att tgt ggg agc aca acc gga ggc ctt ggt ctt ctt gga ctg tac ata   6400
Ile Cys Gly Ser Thr Thr Gly Gly Leu Gly Leu Leu Gly Leu Tyr Ile
   2010                2015                2020

aat gaa aag aat gta gca ctt atc aac caa acc ctg gag agt ctg acg   6448
Asn Glu Lys Asn Val Ala Leu Ile Asn Gln Thr Leu Glu Ser Leu Thr
2025                2030                2035                2040

gag tac tgt caa ggg cct tgc cat gag aac cag aac tgc atc gcc acc   6496
Glu Tyr Cys Gln Gly Pro Cys His Glu Asn Gln Asn Cys Ile Ala Thr
                2045                2050                2055

cac gag tcc aat ggc atc gat atc atc aca gcc ctc atc ctc aat gat   6544
His Glu Ser Asn Gly Ile Asp Ile Ile Thr Ala Leu Ile Leu Asn Asp
           2060                2065                2070

atc aac cct ctg gga aag aag cgg atg gac ctg gtg tta gaa ctg aag   6592
Ile Asn Pro Leu Gly Lys Lys Arg Met Asp Leu Val Leu Glu Leu Lys
      2075                2080                2085

aac aat gct tcg aag ctg cta ctg gcc atc atg gaa agc aga cac gat   6640
Asn Asn Ala Ser Lys Leu Leu Leu Ala Ile Met Glu Ser Arg His Asp
   2090                2095                2100

agt gaa aat gca gag agg atc ctg tac aac atg agg ccc aag gag ctg   6688
Ser Glu Asn Ala Glu Arg Ile Leu Tyr Asn Met Arg Pro Lys Glu Leu
2105                2110                2115                2120

gtg gaa gtg atc aag aag gcc tac atg caa ggt gaa gtg gaa ttt gag   6736
Val Glu Val Ile Lys Lys Ala Tyr Met Gln Gly Glu Val Glu Phe Glu
                2125                2130                2135

gat ggg gag aac ggt gag gat gga gct gcc tca ccc agg aac gtg ggc   6784
Asp Gly Glu Asn Gly Glu Asp Gly Ala Ala Ser Pro Arg Asn Val Gly
           2140                2145                2150

cac aac atc tac atc ctc gct cac cag ttg gct cgg cat aac aaa gaa   6832
His Asn Ile Tyr Ile Leu Ala His Gln Leu Ala Arg His Asn Lys Glu
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2155 2160 2165

ctt caa acc atg ctg aaa cct gga ggc cag gtg gat ggg gat gaa gct 6880
Leu Gln Thr Met Leu Lys Pro Gly Gly Gln Val Asp Gly Asp Glu Ala

2170 2175 2180

ctg gag ttc tac gcg aag cac aca gca caa att gag att gtc aga ctg 6928
Leu Glu Phe Tyr Ala Lys His Thr Ala Gln Ile Glu Ile Val Arg Leu

2185 2190 2195 2200

gac cgg aca atg gaa cag atc gtc ttc cct gtg ccc agc atc tgt gaa 6976
Asp Arg Thr Met Glu Gln Ile Val Phe Pro Val Pro Ser Ile Cys Glu

2205 2210 2215

ttc ctg act aag gaa tcg aaa ctt cga ata tat tac acc aca gag cgg 7024
Phe Leu Thr Lys Glu Ser Lys Leu Arg Ile Tyr Tyr Thr Thr Glu Arg

2220 2225 2230

gat gag caa ggt agc aag atc aat gac ttc ttc ctg cgc tcc gag gac 7072
Asp Glu Gln Gly Ser Lys Ile Asn Asp Phe Phe Leu Arg Ser Glu Asp

2235 2240 2245

ctc ttt aac gag atg aac tgg cag aag aaa ctt cga gcc cag cct gtc 7120
Leu Phe Asn Glu Met Asn Trp Gln Lys Lys Leu Arg Ala Gln Pro Val

2250 2255 2260

ttg tac tgg tgt gcc cga aac atg tct ttc tgg agc agc atc tcc ttc 7168
Leu Tyr Trp Cys Ala Arg Asn Met Ser Phe Trp Ser Ser Ile Ser Phe

2265 2270 2275 2280

aac ctg gcc gtc ctg atg aac ctg ctg gtg gcg ttt ttc tat cca ttt 7216
Asn Leu Ala Val Leu Met Asn Leu Leu Val Ala Phe Phe Tyr Pro Phe

2285 2290 2295

aaa gga gtg agg gga gga aca cta gag cca cac tgg tca ggc ctc ctg 7264
Lys Gly Val Arg Gly Gly Thr Leu Glu Pro His Trp Ser Gly Leu Leu

2300 2305 2310

tgg aca gcc atg ctc atc tct ctg gcc att gtc att gct ctg ccc aag 7312

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Trp Thr Ala Met Leu Ile Ser Leu Ala Ile Val Ile Ala Leu Pro Lys
       2315                2320                2325
ccc cac ggc atc cgg gcc tta att gct tct aca atc cta cga ctg ata   7360
Pro His Gly Ile Arg Ala Leu Ile Ala Ser Thr Ile Leu Arg Leu Ile
   2330                2335                2340
ttt tca gtt ggg ttg cag ccc aca ctg ttt ctg ctg gga gct ttc aat   7408
Phe Ser Val Gly Leu Gln Pro Thr Leu Phe Leu Leu Gly Ala Phe Asn
2345                2350                2355                2360
gtc tgc aat aaa atc atc ttc ctg atg agc ttt gtg ggc aac tgt ggg   7456
Val Cys Asn Lys Ile Ile Phe Leu Met Ser Phe Val Gly Asn Cys Gly
                2365                2370                2375
acc ttc acc aga ggc tac cgg gcc atg gtt ctg gat gtg gag ttc ctc   7504
Thr Phe Thr Arg Gly Tyr Arg Ala Met Val Leu Asp Val Glu Phe Leu
            2380                2385                2390
tat cat ttg ctg tat cta ctc atc tgt gcc atg ggc ctc ttc gta cat   7552
Tyr His Leu Leu Tyr Leu Leu Ile Cys Ala Met Gly Leu Phe Val His
       2395                2400                2405
gag ttc ttc tat agc ttg ctg ctt ttt gat tta gtg tac aga gag gag   7600
Glu Phe Phe Tyr Ser Leu Leu Leu Phe Asp Leu Val Tyr Arg Glu Glu
   2410                2415                2420
act ttg ctt aat gtc att aaa agt gtc acc cgc aat gga cgg tcc atc   7648
Thr Leu Leu Asn Val Ile Lys Ser Val Thr Arg Asn Gly Arg Ser Ile
2425                2430                2435                2440
atc ttg aca gcg gtc ctg gct ctg atc ctg gtt tac ctg ttc tca att   7696
Ile Leu Thr Ala Val Leu Ala Leu Ile Leu Val Tyr Leu Phe Ser Ile
                2445                2450                2455
gtg ggc tat ctg ttc ttc aag gat gac ttt atc ttg gaa gta gat agg   7744
Val Gly Tyr Leu Phe Phe Lys Asp Asp Phe Ile Leu Glu Val Asp Arg
            2460                2465                2470
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ttg ccc aat gaa aca gct gtt cca gaa act ggc gag agt ttg gcc aac 7792
Leu Pro Asn Glu Thr Ala Val Pro Glu Thr Gly Glu Ser Leu Ala Asn
2475 2480 2485

gat ttc ctg tac tct gat gtg tgc agg gta gag acg ggg gag aac tgc 7840
Asp Phe Leu Tyr Ser Asp Val Cys Arg Val Glu Thr Gly Glu Asn Cys
2490 2495 2500

acc tct cct gca ccc aaa gaa gag ctg ctc cct gcc gaa gaa acg gaa 7888
Thr Ser Pro Ala Pro Lys Glu Glu Leu Leu Pro Ala Glu Glu Thr Glu
2505 2510 2515 2520

cag gat aag gaa cac acg tgt gag acc ctg ctc atg tgc atc gtc act 7936
Gln Asp Lys Glu His Thr Cys Glu Thr Leu Leu Met Cys Ile Val Thr
2525 2530 2535

gtt ctg agt cac ggg ctg cgg agt ggg gga ggg gta gga gac gtg ctc 7984
Val Leu Ser His Gly Leu Arg Ser Gly Gly Gly Val Gly Asp Val Leu
2540 2545 2550

agg aag cca tcc aaa gag gag cct ctg ttt gct gca agg gtg atc tac 8032
Arg Lys Pro Ser Lys Glu Glu Pro Leu Phe Ala Ala Arg Val Ile Tyr
2555 2560 2565

gac ctc ctc ttc ttc ttc atg gtc atc atc atc gtc ctg aac ctg att 8080
Asp Leu Leu Phe Phe Phe Met Val Ile Ile Ile Val Leu Asn Leu Ile
2570 2575 2580

ttc ggg gtc atc atc gac acc ttt gct gac ctg agg agt gag aag caa 8128
Phe Gly Val Ile Ile Asp Thr Phe Ala Asp Leu Arg Ser Glu Lys Gln
2585 2590 2595 2600

aag aag gag gag atc tta aaa acc acg tgc ttc atc tgc ggc ttg gaa 8176
Lys Lys Glu Glu Ile Leu Lys Thr Thr Cys Phe Ile Cys Gly Leu Glu
2605 2610 2615

agg gac aag ttt gac aat aag act gtc acc ttt gaa gag cac atc aag 8224
Arg Asp Lys Phe Asp Asn Lys Thr Val Thr Phe Glu Glu His Ile Lys

2620 2625 2630

gaa gaa cac aac atg tgg cac tat ctg tgc ttc atc gtg ctg gtg aaa 8272
Glu Glu His Asn Met Trp His Tyr Leu Cys Phe Ile Val Leu Val Lys

2635 2640 2645

gtg aag gac tcc aca gag tac acc ggg cct gag agt tac gtg gca gag 8320
Val Lys Asp Ser Thr Glu Tyr Thr Gly Pro Glu Ser Tyr Val Ala Glu

2650 2655 2660

atg atc agg gaa aga aac ctt gat tgg ttc ctc aga atg aga gcc atg 8368
Met Ile Arg Glu Arg Asn Leu Asp Trp Phe Leu Arg Met Arg Ala Met

2665 2670 2675 2680

tcc ctg gtc agc agc gat tct gaa ggg gaa cag aac gag ctg agg aac 8416
Ser Leu Val Ser Ser Asp Ser Glu Gly Glu Gln Asn Glu Leu Arg Asn

2685 2690 2695

ctg cag gag aag ctg gag tct acc atg aag ctg gtc acc aat ctt tct 8464
Leu Gln Glu Lys Leu Glu Ser Thr Met Lys Leu Val Thr Asn Leu Ser

2700 2705 2710

ggc cag ctg tca gaa cta aag gac cag atg aca gaa cag agg aag cag 8512
Gly Gln Leu Ser Glu Leu Lys Asp Gln Met Thr Glu Gln Arg Lys Gln

2715 2720 2725

aaa caa aga atc ggc ctt cta gga cat cct cct cac atg aat gtc aac 8560
Lys Gln Arg Ile Gly Leu Leu Gly His Pro Pro His Met Asn Val Asn

2730 2735 2740

cca cag cag ccg gcc taggcaaatg aggcagaggg actctgctca gccctctgta 8615
Pro Gln Gln Pro Ala

2745

tatcactgtc agggtgggta cggctcattg gttctgattt gcccactaag ggtacatgtg 8675

cgcttagtac atttgtaaat actcagtttt gtattgtatg tatatgattg ctattctcag 8735

aggtttggac tttcgtattg taattagctc tgttggcatg gtgacttgtc actcctgcca 8795

aaaatattaa aaatgccttt tttggaagga ctacagaaag tacctgattt gcacttgaac 8855

cagattatag atttaaaagt atatgacatg tattttgtat ttaaaactag aatagccagt 8915

atttatgttt tttataaaac tgtgcaatac aaattatgca atcaccataa ctttgtaact 8975

cctgagtgtc ctaagggagt acacatcttt gaagctgatt tgttgatact cgtgtaataa 9035

atggttaaat atcaaatgct gctgctgctg ccaaaattat attaatagcg agtttctggc 9095

ccctgggcaa ttttgtacct tgtaattatc ctatggtgat gctgtttctc gttgctaatg 9155

gcattagtgc ccctgtatcc tagtgataac tccaggtctg tgaaccattc aaacagcatt 9215

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aaagtgtttc tttaagagag ccatgttaga ggctcacact ttagcttgaa aggagttgat 9335

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Arg Tyr Ser Ala Gln Lys Gln Phe Trp Lys Ala Ala Lys Pro Gly Ala
65                  70                  75                  80
Asn Ser Thr Thr Asp Ala Val Leu Leu Asn Lys Leu His His Ala Ala
                85                  90                  95
Asp Leu Glu Lys Lys Gln Asn Glu Thr Glu Asn Arg Lys Leu Leu Gly
            100                 105                 110
Thr Val Ile Gln Tyr Gly Asn Val Ile Gln Leu Leu His Leu Lys Ser
        115                 120                 125
Asn Lys Tyr Leu Thr Val Asn Lys Arg Leu Pro Ala Leu Leu Glu Lys
    130                 135                 140
Asn Ala Met Arg Val Thr Leu Asp Glu Ala Gly Asn Glu Gly Ser Trp
145                 150                 155                 160
Phe Tyr Ile Gln Pro Phe Tyr Lys Leu Arg Ser Ile Gly Asp Ser Val
                165                 170                 175
Val Ile Gly Asp Lys Val Val Leu Asn Pro Val Asn Ala Gly Gln Pro
            180                 185                 190
Leu His Ala Ser Ser His Gln Leu Val Asp Asn Pro Gly Cys Asn Glu
        195                 200                 205
Val Asn Ser Val Asn Cys Asn Thr Ser Trp Lys Ile Val Leu Phe Met
    210                 215                 220
Lys Trp Ser Asp Asn Lys Asp Asp Ile Leu Lys Gly Gly Asp Val Val
225                 230                 235                 240
Arg Leu Phe His Ala Glu Gln Glu Lys Phe Leu Thr Cys Asp Glu His
                245                 250                 255
Arg Lys Lys Gln His Val Phe Leu Arg Thr Thr Gly Arg Gln Ser Ala
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Phe Lys His Leu Ala Thr Gly His Tyr Leu Ala Ala Glu Val Asp Pro

305 310 315 320

Asp Phe Glu Glu Glu Cys Leu Glu Phe Gln Pro Ser Val Asp Pro Asp

325 330 335

Gln Asp Ala Ser Arg Ser Arg Leu Arg Asn Ala Gln Glu Lys Met Val

340 345 350

Tyr Ser Leu Val Ser Val Pro Glu Gly Asn Asp Ile Ser Ser Ile Phe

355 360 365

Glu Leu Asp Pro Thr Thr Leu Arg Gly Gly Asp Ser Leu Val Pro Arg

370 375 380

Asn Ser Tyr Val Arg Leu Arg His Leu Cys Thr Asn Thr Trp Val His

385 390 395 400

Ser Thr Asn Ile Pro Ile Asp Lys Glu Glu Glu Lys Pro Val Met Leu

405 410 415

Lys Ile Gly Thr Ser Pro Leu Lys Glu Asp Lys Glu Ala Phe Ala Ile

420 425 430

Val Pro Val Ser Pro Ala Glu Val Arg Asp Leu Asp Phe Ala Asn Asp

435 440 445

Ala Ser Lys Val Leu Gly Ser Ile Ala Gly Lys Leu Glu Lys Gly Thr

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    530                 535                 540
Arg His Ala Pro Phe Arg His Ile Cys Arg Leu Cys Tyr Arg Val Leu
545                 550                 555                 560
Arg His Ser Gln Gln Asp Tyr Arg Lys Asn Gln Glu Tyr Ile Ala Lys
                565                 570                 575
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            580                 585                 590
Asp Thr Ile Thr Ala Leu Leu His Asn Asn Arg Lys Leu Leu Glu Lys
        595                 600                 605
His Ile Thr Ala Ala Glu Ile Asp Thr Phe Val Ser Leu Val Arg Lys
    610                 615                 620
Asn Arg Glu Pro Arg Phe Leu Asp Tyr Leu Ser Asp Leu Cys Val Ser
625                 630                 635                 640
Met Asn Lys Ser Ile Pro Val Thr Gln Glu Leu Ile Cys Lys Ala Val
                645                 650                 655
Leu Asn Pro Thr Asn Ala Asp Ile Leu Ile Glu Thr Lys Leu Val Leu
            660                 665                 670
Ser Arg Phe Glu Phe Glu Gly Val Ser Thr Gly Glu Asn Ala Leu Glu
        675                 680                 685
Ala Gly Glu Asp Glu Glu Glu Val Trp Leu Phe Trp Arg Asp Ser Asn
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740 745 750

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755 760 765

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770 775 780

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785 790 795 800

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820 825 830

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835 840 845

Cys Gln Arg Phe Pro Phe Ser Asp Lys Glu Lys Asn Lys Leu Thr Phe

850 855 860

Glu Val Val Asn Leu Ala Arg Asn Leu Ile Tyr Phe Gly Phe Tyr Asn

865 870 875 880

Phe Ser Asp Leu Leu Arg Leu Thr Lys Ile Leu Leu Ala Ile Leu Asp

885 890 895

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900 905 910

Glu Glu Asn Lys Gly Ser Asn Val Met Arg Ser Ile His Gly Val Gly

915 920 925

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930 935 940

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945 950 955 960

Glu Lys Glu Asp Ile Met Val Met Asp Thr Lys Leu Lys Ile Ile Glu
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995 1000 1005

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825 1830 1835 1840

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1845 1850 1855

Lys Val Phe Tyr Asp Arg Met Lys Val Ala Gln Gln Glu Ile Lys Ala

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2005 2010 2015

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Trp Phe Leu Arg Met Arg Ala Met Ser Leu Val Ser Ser Asp Ser Glu
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Met Lys Leu Val Thr Asn Leu Ser Gly Gln Leu Ser Glu Leu Lys Asp
705 2710 2715 2720

Gln Met Thr Glu Gln Arg Lys Gln Lys Gln Arg Ile Gly Leu Leu Gly
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Claims

1. A recombinant polypeptide of the following (a), (b) or (c):

   (a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO: 2;
   (b) a polypeptide comprising an amino acid sequence having deletion, substitution or addition of at least one amino acid in the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity to inositol 1,4,5-trisphosphate; or
   (c) a polypeptide having at least 70% homology with the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity with inositol 1,4,5-trisphosphate.

2. A gene coding for a polypeptide of the following (a), (b) or (c):

   (a) a polypeptide comprising an amino acid sequence shown in SEQ ID NO: 2;
   (b) a polypeptide comprising an amino acid sequence having deletion, substitution or addition of at least one amino acid in the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity with inositol 1,4,5-trisphosphate; or
   (c) a polypeptide having at least 70% homology with the amino acid sequence shown in SEQ ID NO: 2, and having a high affinity binding activity to inositol 1,4,5-trisphosphate.

3. A gene comprising DNA of the following (d) or (e):

   (d) DNA of a nucleotide sequence shown in SEQ ID NO: 1; or
   (e) DNA of a nucleotide sequence having at least 70% homology with the DNA of the nucleotide sequence shown in SEQ ID NO: 1, and coding for a polypeptide having a high affinity binding activity with inositol 1,4,5-trisphosphate.

4. A gene comprising DNA which has a nucleotide sequence having at least 70% homology with the gene of claim 2, and which codes for a polypeptide having a high affinity binding activity with inositol 1,4,5-trisphosphate.

5. A recombinant vector comprising the gene of any one of claims 2 to 4.

6. A transformant comprising the recombinant vector of claim 5.

7. A method for producing the polypeptide of any one of the preceding claims, comprising:

   culturing the transformant of claim 6; and
   collecting, from the obtained culture, a polypeptide having a high affinity binding activity to inositol 1,4,5-trisphosphate.

8. An antagonist for IP_3-induced calcium comprising the protein of claim 1.

9. A therapeutic agent diseases associated with calcium production comprising the protein of claim 1.

10. An agent for gene therapy for diseases associated with calcium production comprising the gene of claims 2 or 3.

11. The therapeutic agent of claims 10 or 11, wherein the disease is at least one disease selected from the group consisting of diseases in the nervous system, blood vascular system, respiratory system, digestive system, lymphatic system, urinary system and reproduction system.

FIG. 1

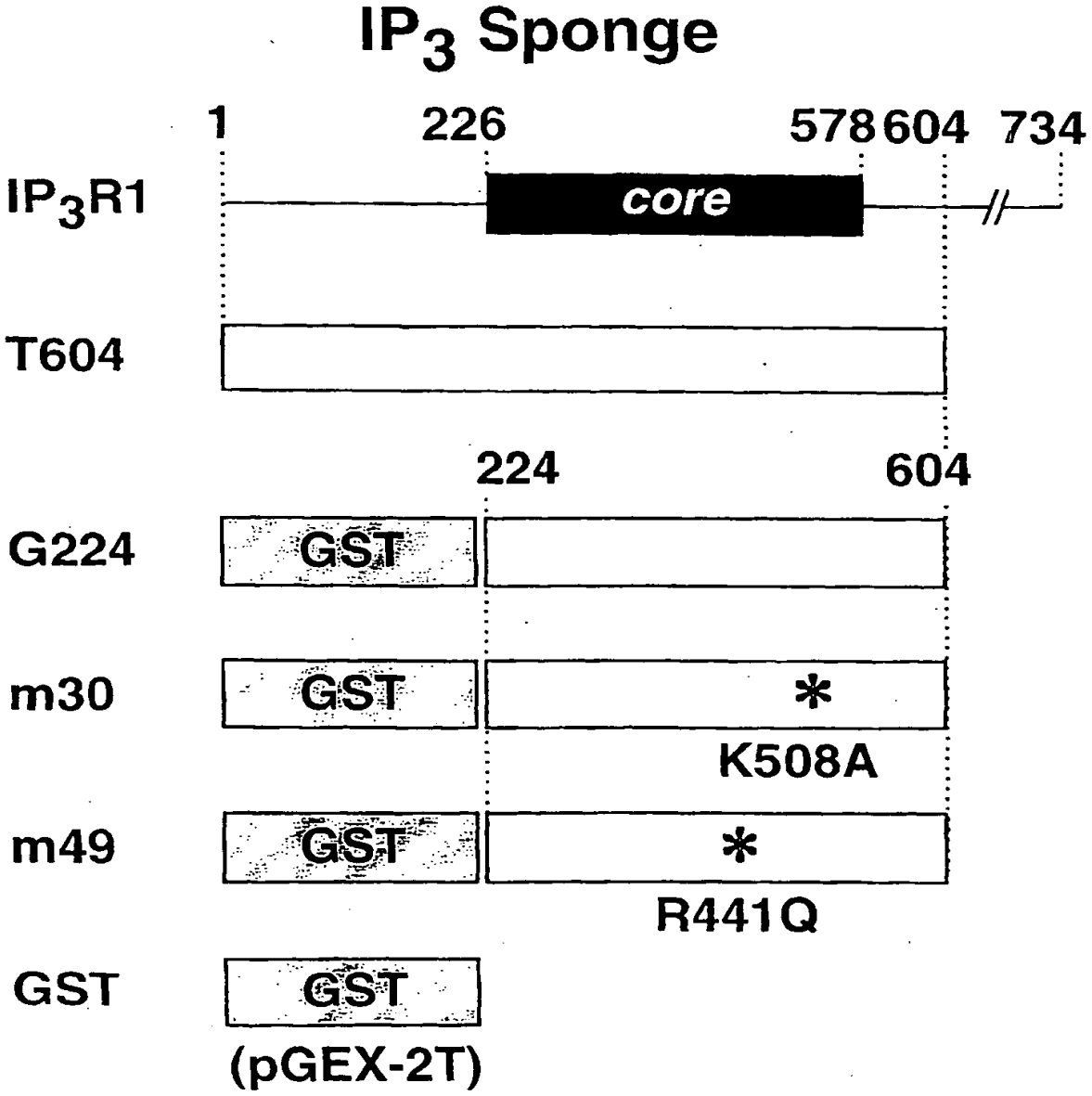


FIG. 2C

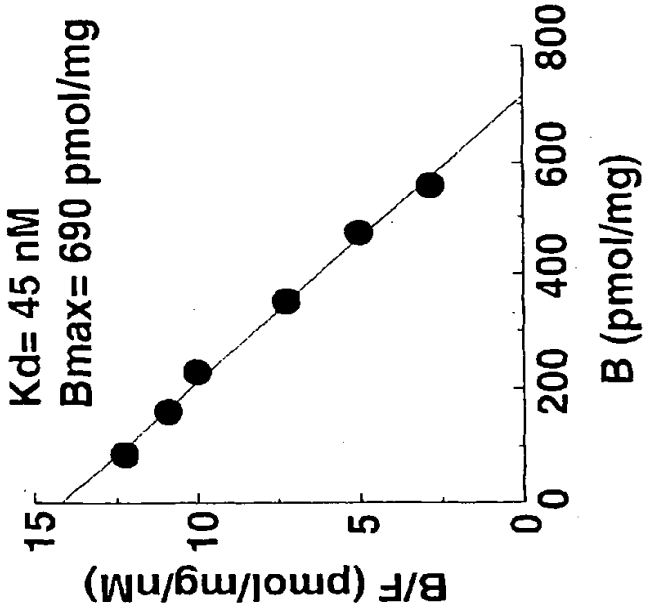


FIG. 2B

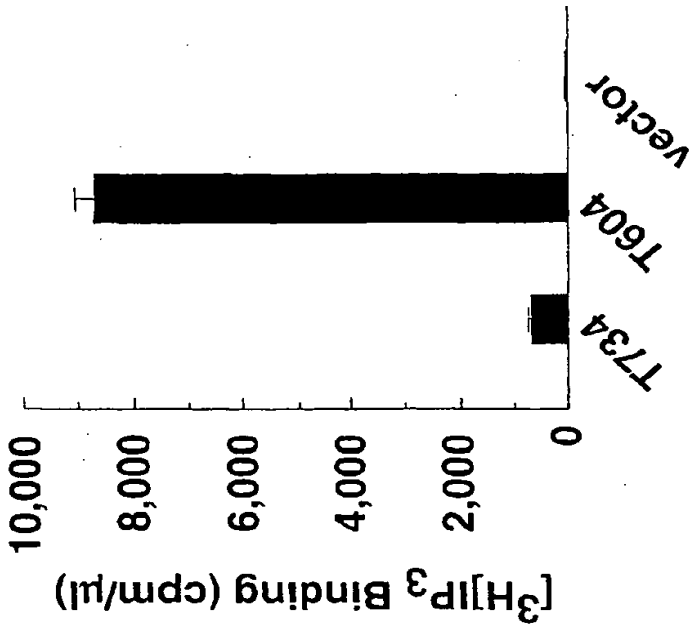


FIG. 2A

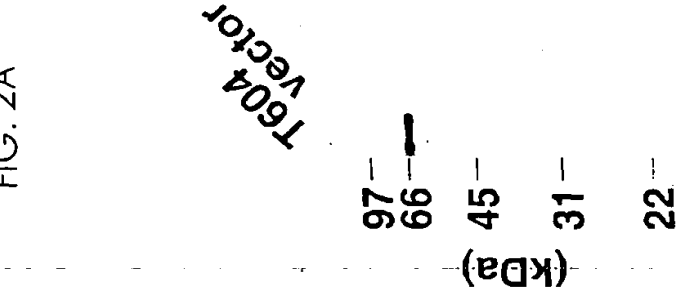


FIG. 3A

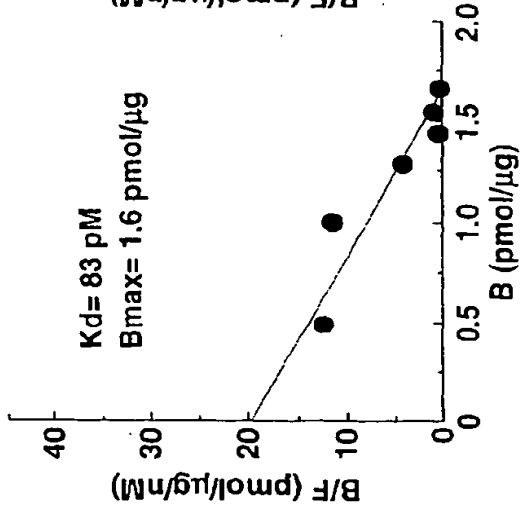


FIG. 3B

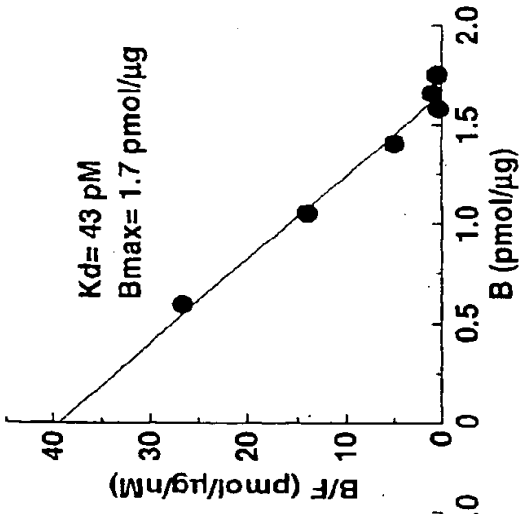
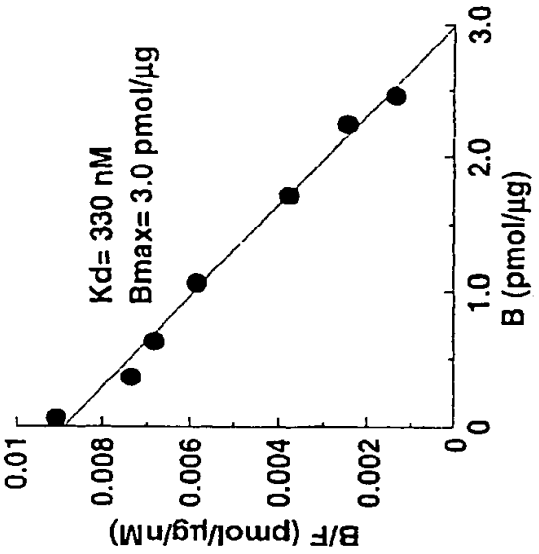


FIG. 3C



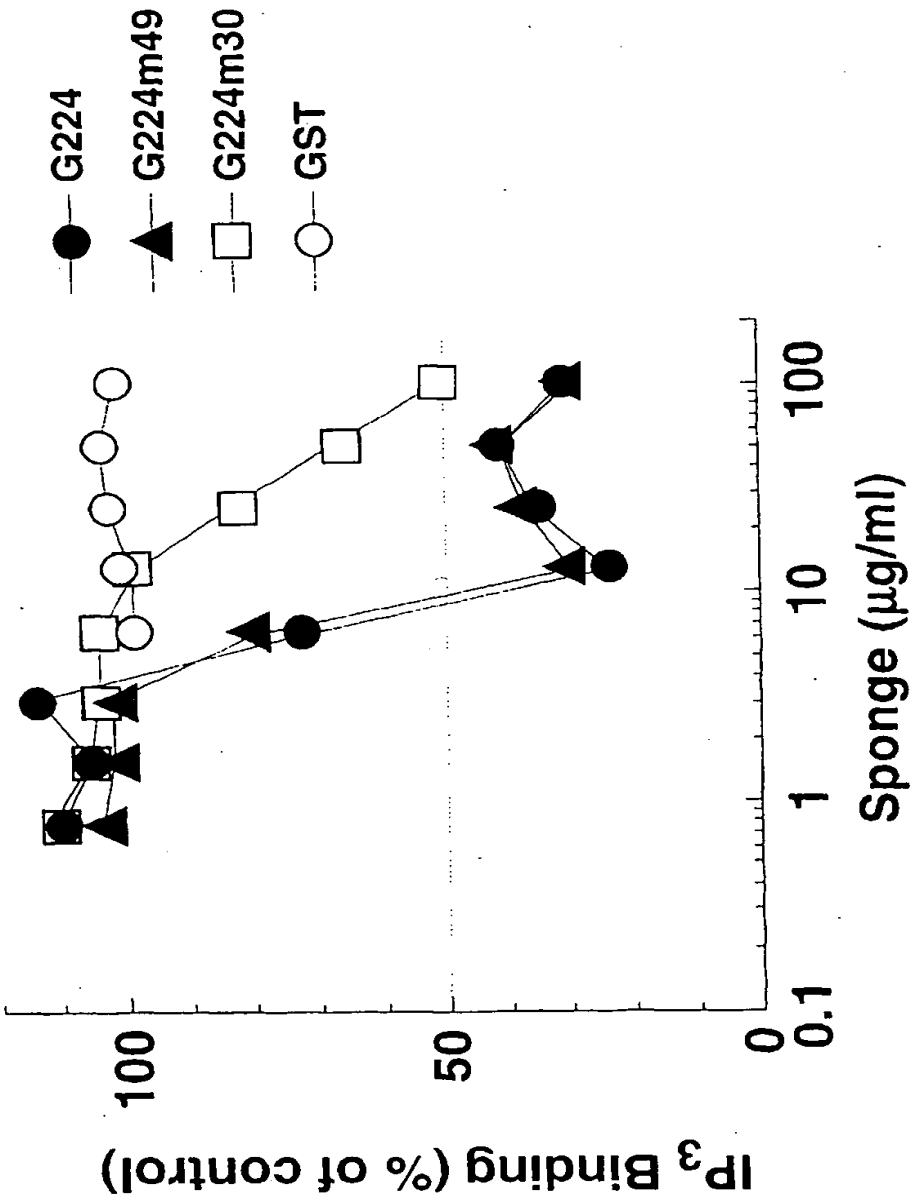

G224
G224m49
G224m30
GST
IP3 Binding (% of control)
100
50
0
0.1
1
10
100
Sponge (µg/ml)

FIG. 4

FIG. 5A

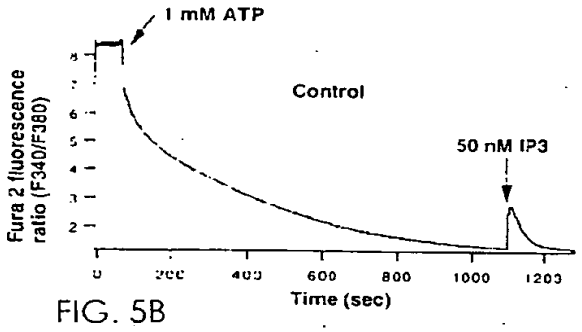


FIG. 5B

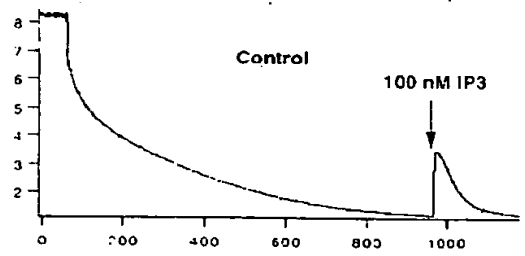


FIG. 5C

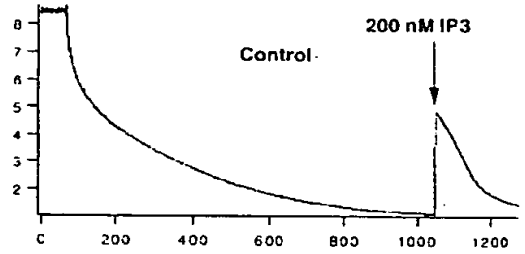


FIG. 5D

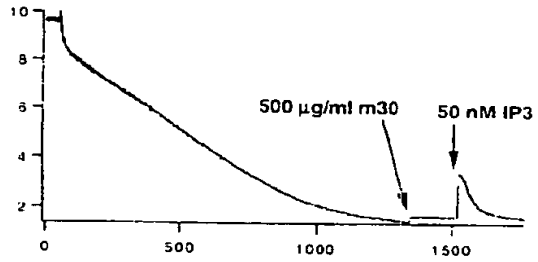


FIG. 5E

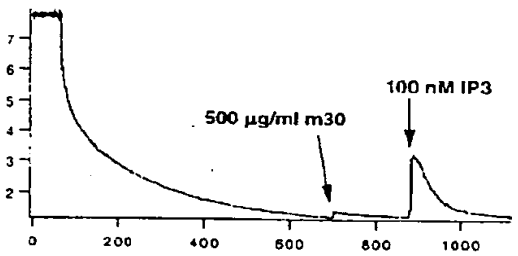


FIG. 5F

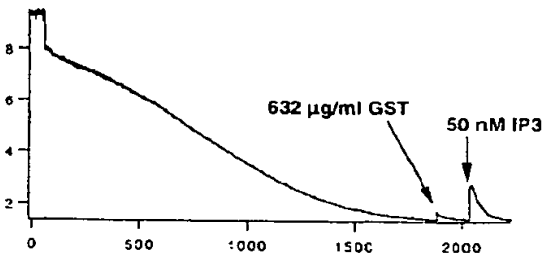


FIG. 6A

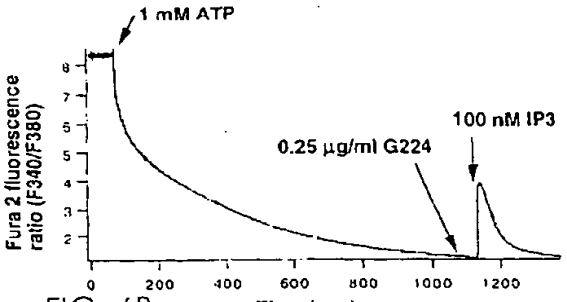


FIG. 6B

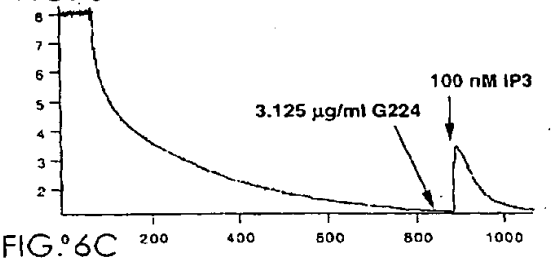


FIG. 6C

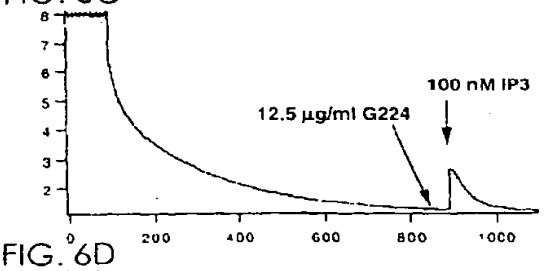


FIG. 6D

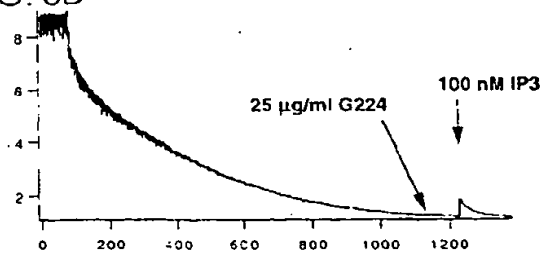


FIG. 6E

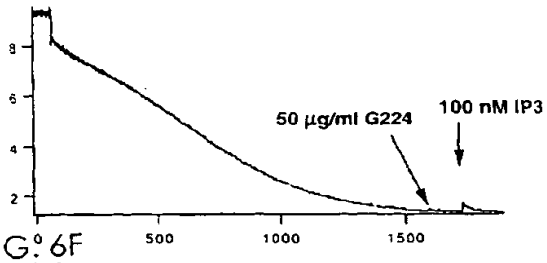


FIG. 6F

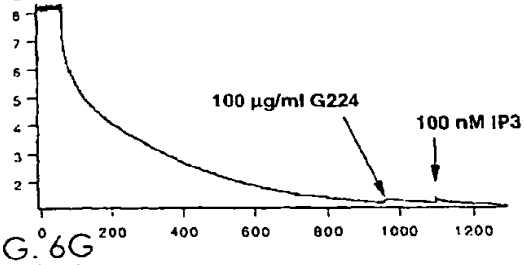


FIG. 6G

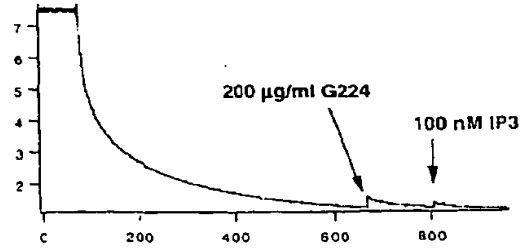
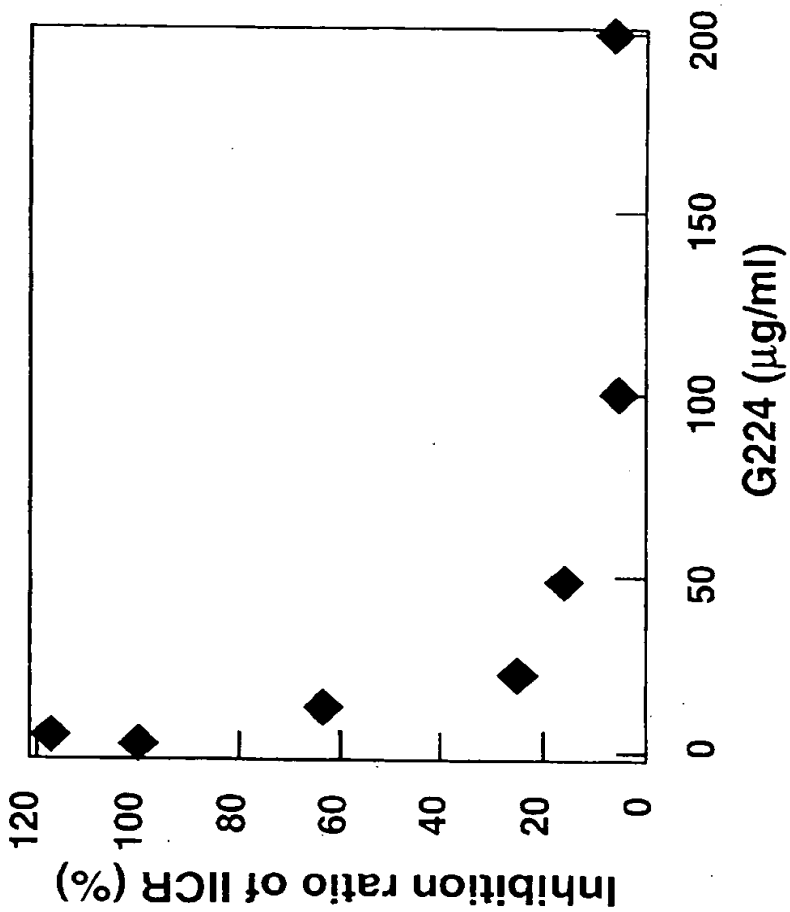


FIG. 7



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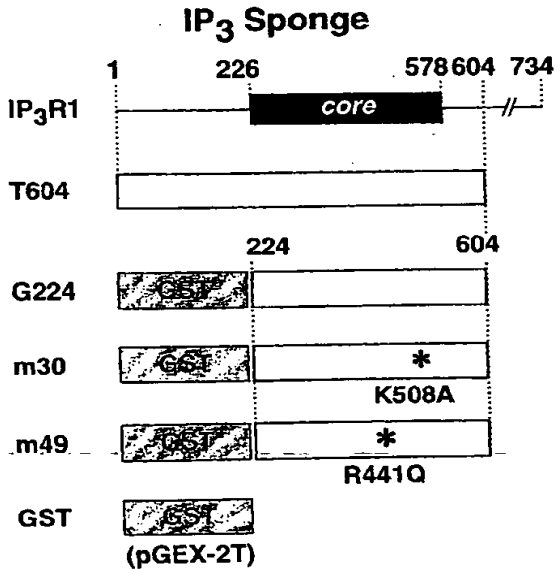
(54) High affinity IP3-binding polypeptide

(57) The present invention provides a high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate, to a gene encoding the polypeptide, to a recombinant vector including the gene, to a transformant including the vector and to a method for producing the high affinity polypeptide having a binding activity to inositol 1,4,5-trisphosphate.

FIG. 1



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EUROPEAN SEARCH REPORT

Application Number
EP 99 30 6879

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	FURUICHI T ET AL.: "Primary structure and functinal expression of the inositol 1,4,5-trisphosphate-binding protein P400" NATURE, vol. 342, 2 November 1989 (1989-11-02), pages 32-38, XP002141737 * the whole document * ---	1-7	C12N15/12 C07K14/705 C12N1/20 C12N15/62 A61K38/17 A61K48/00
X	WO 96 24846 A (SINAI SCHOOL MEDICINE) 15 August 1996 (1996-08-15) * the whole document * ---	1-7	
X	DATABASE WPI Week 9648 Derwent Publications Ltd., London, GB; AN 1996-482258 XP002141738 "Human type I tri-phosphate receptor" & JP 08 245698 A (SOSEI KK), 24 September 1996 (1996-09-24) * abstract * ---	1-9	
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	4 July 2000	Oderwald, H

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